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FOURTH (FINAL) REPORT ON A TEST OF McDOUGALL'S LAMARCKIAN EXPERIMENT ON THE TRAINING OF RATS

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INTRODUCTION

This is the final report on the experiment, begun by us in 1932, and of which three interim reports have already been published (1935, 1942, 1948). It was essentially an examination of the well-known experiment of McDougall, purporting to have demonstrated a Lamarckian effect in the inheritance of an induced light phobia in rats.

Our experiment consisted in placing the rats into a tank of water from which they emerged by the choice of one of two exits. Of these one was illuminated, the other not; and a preference for the non-illuminated (dim) exit was induced in the rats by electrifying the illuminated exit. With this apparently simple problem of learning to avoid the lighted exit, the rats were daily confronted until they learnt to solve it. The number of errors made by the rats was recorded and a sustained diminution in the number of these errors in successive generations, measured against a control series of generations, is the criterion for the operation of a Lamarckian factor.

Over the thirty-two generations of McDougall's experiment, for which records are available, there was such a progressive decline in the number of errors. McDougall attributed this improvement in facility in learning to the inheritance of the effects of ancestral training. At the time, this conclusion was justified to the extent that no alternative explanation could be advanced to account for it. It was this that led us, and Crew, to repeat the experiment. Crew (1936) found no evidence of increased facility in learning during the eighteen generations of his experiment.

TRAINING PROCEDURE

We will give a brief description of the methods we have used; fuller details are to be found in our First Report. The apparatus was essentially as designed by McDougall (1930). It consisted (Fig. 1) of a tank of water divided into three parallel passages communicating with one another at the far curved end of the tank. At the near end of each side-passage was a sloping wire ramp up which the rats could scramble from the water. Behind a sheet of ground glass at the back of each ramp was an electric lamp which shone down the passage and illuminated its

communication with the central passage. The circuit was arranged so that one or the other lamp could be lit alternately. Coupled with the lighting circuit was a second circuit that electrified the ramp on the illuminated side; a current of 230 V., 1·2 mA. was used, with a duration of 3 sec.

A rat placed in the water at the near end of the central passage swam along it and then had a choice of two escape routes. If it chose the bright ramp it escaped at the expense of a 3 sec. electric shock. The rat had to learn to escape always by the dim exit, irrespective of whether this was on the right side or the left. Facility in learning was measured by the number of errors made, i.e. the number of escapes by the bright exit, before it learnt to use the dim one always. A rat was held to have learnt the task as soon as it made twelve consecutive correct runs.

Our routine procedure has been to wean the rats when 26 days old. To acquaint

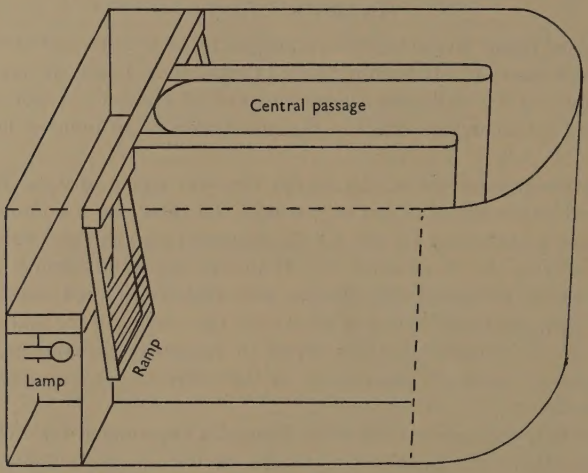

Fig. 1. Diagram of training tank.

them with the training apparatus, they were given, on the following day, six 'runs' without illuminated ramps, after which normal training began. This consisted of four runs per day for 5 days (the animals still being rather small) and thereafter six per day until the task was learnt. A small proportion of rats, that had failed to solve the problem by the 52nd day of training, were given 'special training'. They were, almost without exception, rats that had developed the habit of going exclusively to one ramp and were quite unable to solve the problem because of unawareness of the alternative exit. 'Special training' consisted in forcing the animals, usually against strong resistance, to take the correct pathway. Training of all rats was continued after learning was complete, and until the time of mating, but was limited to two runs per day.

CONTROLS

A fundamental weakness in McDougall's experiment was his failure to maintain a control line of rats for comparison with his trained line. In our experiment we instituted a proper control line, bred parallel with the trained line and under the same conditions. All the rats were descendants of a single pair of Wistar origin. The first generation obtained from this pair (which was not trained) was divided into two groups, one of which was trained and became the ancestors of the trained line (*T*). The other group was not trained and became the ancestors of the control line (*C*). In each generation the required number of rats of the trained line was trained and mated as parents of the next generation. In the control line some litters were not trained but were kept as parents of the next generation; other litters of this line were trained to provide controls to the same generation of the trained line. These trained controls were, of course, not used for breeding. In this way each generation of the trained line was tested against an approximately equal number of controls, differing from the trained line only in the fact that their ancestors were not trained.

In our Third Report we stated that genetic differences in colour pattern and body size between the trained and control lines had appeared, and suggested that mutations could have been responsible for the consistent superiority of the trained line over the control between about generations 12–28. This raised the possibility that further mutations, having a direct effect on the rate of learning, might occur. If such mutations accumulated in the trained line their effect would simulate that of Lamarckian inheritance. To meet this possibility we took the precaution of maintaining from generation 41 onwards, two trained sublines (*TA* and *TB*) and two control sublines (*CA* and *CB*). The offspring of generation 40 of the trained line were divided into two groups. One became the ancestors of subline *TA*, the other, the ancestors of subline *TB*. The controls were treated in a similar manner. The two control sublines were thus joint controls to the two trained sublines; there was no special relationship between *TA* and *CA*, or between *TB* and *CB*. It may be stated at once that there was no evidence of divergence, in respect of facility in learning, between the sublines during the ten generations, 41–50.

MATING AND MORTALITY

The minimum age at which the rats were mated was 85 days. By this time even the rats which required 'special training' had learnt the task. In order that every rat, whether it had learnt quickly or slowly, should have an equal chance of becoming a parent, all the rats of a generation were mated at the same time.

The rats were mated without reference to their training scores. Except for a short period, brother-sister matings were avoided as far as possible. Not all the mated rats became parents of the next generation, for many of the matings proved infertile, and others did not produce litters till after the number of young required had been obtained. This, of course, applied to both the trained and control lines.

Of the 4654 rats which started training forty-three died before they had learnt the task. These forty-three rats have been excluded from our figures. Throughout the whole of the experiment there were no injuries of any kind attributable to the electric shock.

MEASURE OF PERFORMANCE

In previous reports we have discussed the problem of finding a satisfactory measure of the performance of a group of rats as a whole. The use of the arithmetic mean number of errors made by the rats is unsatisfactory owing to the extreme skewness of the distribution (Second Report, Table 1) and, in any case, is invalidated by our practice of giving 'special training' to the very slow learners. In this report, and for the analyses of the results of the experiment, we have used the measure adopted in our Third Report: 'The scores of the first thousand control rats were arranged in order of magnitude, and the whole group divided into ten classes, each containing as nearly as possible an equal number of rats, having regard to the fact that the number of errors are necessarily whole numbers.'

The resulting distribution is shown in Table 1.

Table 1. *The first* 1000 *controls classified according to the number of errors made*

Class ...	1	2	3	4	5	6	7	8	9	10
No. of errors	0–5	6–10	11–15	16–19	20–23	24–27	28–32	33–40	41–67	68–
No. of rats	93	110	102	105	93	96	106	95	99	101

Thus all the rats with training scores 0–5 errors inclusive are placed in class 1, and so on. The arithmetic mean of the classes so obtained will be referred to as the *mean class* of the group of rats concerned.

BODY SIZE IN THE TRAINED AND CONTROL LINES

In our Third Report we discussed a genetic difference in body size between the trained line and the control. Weighings made in generations 25–28, and also in generations 34–36, showed that the rats of the trained line were substantially heavier than the controls. At 26 days old the difference in mean weights was approximately 13 g. (Table 2).

Further series of weighings at 26 days old were made in generations 49 and 50. These showed (Table 2) that there was no difference in weight between the two trained sublines, but that the mean weights, by comparison with those of generations previously weighed, had fallen by about 9 g. The mean weights were: females (85) 43·6 g., males (70) 45·2 g. Only 6% of the rats in these two generations weighed more than 50 g.; in generations 25–28 and 34–36, 60% exceeded this weight.

In the controls, the mean weights of the two sublines in generation 49 were much the same. They conformed to those of earlier generations of controls and this was also true of subline *CA* of generation 50. However, in subline *CB* of this generation

the mean weights were: females (23) 43·2 g., males (23) 43·8 g. These are practically the same as those of the trained sublines.

Greenman & Duhring (1931) have recorded the weights of a large number of rats from the Wistar Institute colony. Over a period of 4 years, eight groups of males and females were weighed. At 25 days old, the mean weights of the groups varied from 34·3 to 48·6 g. If 2·5 g. is added to the figures of Greenman & Duhring to allow for the fact that they refer to rats 1 day younger than ours, the total mean weights become: females (423) 43·2 g., males (455) 43·8 g.

Thus, at the end of our experiment there was no evidence of the genetic difference in body size which previously had distinguished the rats of the trained line from the controls and also from Wistar Institute stocks.

Table 2. *Mean weight in grams, with standard errors, of rats at 26 days. The figures in brackets are the number of rats weighed*

Generation	Trained line	Control line
25–28	♀ (73) 50·5±0·5	(153) 38·0±0·4
	♂ (72) 50·4±0·6	(139) 38·7±0·4
34–36	♀ (50) 53·0±0·7	(50) 38·0±0·7
	♂ (50) 54·8±0·7	(50) 40·3±0·7
49*A*	♀ (17) 42·6±0·7	(40) 36·1±0·6
	♂ (12) 44·5±1·4	(29) 37·9±0·6
49*B*	♀ (21) 43·0±0·6	(30) 36·4±0·7
	♂ (14) 43·6±0·8	(23) 38·3±0·7
50*A*	♀ (21) 43·3±1·0	(21) 38·0±1·0
	♂ (20) 43·6±1·0	(24) 39·8±0·9
50*B*	♀ (26) 45·2±0·8	(23) 43·2±0·6
	♂ (24) 47·9±1·0	(23) 43·8±0·5

GENERAL RESULTS OF EXPERIMENT

Data covering generations 1–36 are given in our earlier reports. Tables 3 and 4 of the present report give the data for generations 37–50 which conclude the experiment. The results are summarized in two graphs (Figs. 2, 3). Fig. 2 shows the annual performances over the 20 years of the experiment; Fig. 3 gives the performances of successive generations. In the latter figure, we have arranged the generations as nearly as possible in groups of 4 (see Table 4), in order to minimize chance fluctuations.*

The general result is that periods of progressively decreasing scores have alternated with periods of progressively increasing scores, and in this the controls have participated. Thus there was a fairly regular decrease in the number of errors during the first sixteen generations, a slight increase in the following four and then a further decrease until the twenty-eighth. Over the next eight generations there was a marked increase in the number of errors, and high scores were maintained until the 40th generation after which a further decrease occurred. Thus in spite of the great improvement during the first half of the experiment the scores of the

* The last group necessarily contains only two generations (49 and 50) and in the first group of controls only generations 2–4 are, of course, included.

Table 3. *The number of errors made (shocks received) by each rat, the median number of errors, and the mean class, in each of generations* 37–50

(*T*, trained line; *C*, control line. *S* indicates that the rat qualified for special training. In the trained line the rats which became parents of the next generation are in heavy type.)

Generations	No. of rats	Median	Mean class	No. of errors made by each rat
37 *T*	50	29	6·48	5, 5, 8, 9, **15**, 16, 17, 18, **19**, **19**, **21**, 21, **23**, 23, 25, 25, **27**, 27, 27, 27, 28, **29**, **29**, 29, 29, 29, **30**, **30**, 30, 30, **31**, **32**, **34**, 34, 34, 35, 37, 38, 38, **40**, **40**, 40, 43, **47**, 52, 54, **58**, 91, **99**, *S*
C	50	27·5	6·48	2, 6, 10, 11, 12, 13, 14, 14, 17, 17, 18, 18, 19, 19, 19, 21, 22, 23, 23, 24, 25, 25, 25, 27, 27, 28, 28, 29, 33, 34, 36, 38, 38, 40, 41, 44, 44, 51, 53, 56, 59, 65, 67, 84, *S*, *S*, *S*, *S*, *S*, *S*
38 *T*	50	27·5	6·16	9, 10, 11, 11, **12**, **14**, 14, 15, **16**, **18**, **19**, 19, 19, 20, 20, **21**, 22, 23, 23, 24, 25, 25, 26, 26, 27, 28, 29, **30**, 30, **31**, **31**, **32**, 32, 32, 32, 33, 34, 36, 36, 38, 39, 41, **42**, 45, 47, 49, 50, 53, 56, 124
C	50	26·5	6·32	4, 5, 7, 8, 8, 9, 10, 12, 12, 16, 18, 20, 21, 21, 22, 22, 22, 22, 23, 24, 24, 25, 25, 26, 26, 27, 28, 30, 31, 32, 32, 33, 35, 38, 39, 41, 41, 42, 44, 46, 47, 49, 53, 59, 97, 126, *S*, *S*, *S*, *S*
39 *T*	50	21	5·00	**2**, 2, 2, 2, 3, 4, **5**, 7, 8, **9**, 9, 9, 10, 11, 11, 12, 12, **14**, 15, 17, 17, 17, 20, **21**, 21, 21, **22**, 22, **27**, 27, **28**, 30, **31**, 31, 32, 32, 34, 34, 35, 36, 36, 38, 38, 38, 39, 40, **46**, 54, 60, 93
C	50	22	5·88	6, 8, 9, 11, 13, 14, 14, 15, 15, 16, 16, 16, 16, 17, 17, 17, 18, 18, 18, 19, 19, 21, 21, 21, 22, 22, 23, 25, 25, 29, 29, 30, 31, 32, 33, 34, 34, 36, 41, 41, 42, 46, 48, 57, 67, 74, 76, 93, 108, 118
40 *T*	49	25	5·69	**7**, 8, **11**, 11, 11, 11, 13, **14**, 14, 15, 15, 16, 17, **18**, 19, 19, 19, 20, **21**, 21, 22, 23, 24, 25, 25, 26, 27, 28, 28, 28, **30**, 30, 30, 30, 30, **31**, **31**, **31**, **32**, **33**, **35**, 37, 39, 39, 40, 44, 48, **55**, 75
C	33	22	5·24	6, 6, 8, 8, 9, 10, 11, 12, 12, 13, 13, 14, 15, 16, 20, 20, 22, 23, 23, 25, 26, 29, 31, 31, 32, 33, 36, 38, 47, 54, 55, 69, *S*
41 *TA*	35	23	5·46	2, 5, 6, 9, 10, 11, 11, 11, **12**, 12, 13, 14, 16, 18, **20**, 20, 21, 23, 24, 25, 25, 26, **27**, **29**, 29, 32, **34**, **49**, 52, 53, **54**, 54, **68**, 75, **119**
TB	36	16·5	4·14	1, **3**, 3, 4, 4, 5, 6, 6, 8, **9**, **9**, 9, 9, 14, 15, 15, **16**, 16, **17**, 17, 18, 18, **19**, 19, 20, **21**, 26, **27**, 27, **32**, 32, **43**, **46**, 46, 48, 97
CA	30	20	4·63	4, 11, 11, 12, 13, 13, 13, 14, 14, 14, 14, 15, 16, 17, 19, 21, 21, 21, 21, 22, 22, 23, 25, 26, 26, 29, 32, 40, 48, 49
CB	30	22	5·53	2, 10, 12, 13, 14, 14, 15, 15, 15, 15, 17, 17, 19, 20, 22, 22, 26, 27, 28, 29, 30, 32, 34, 39, 41, 44, 52, 57, 82, 105
42 *TA*	34	15·5	3·94	**3**, **5**, **5**, 6, **7**, 7, **8**, 8, 8, 10, 12, 12, 14, 15, 15, 15, 15, **16**, 16, 17, 18, 18, 19, 21, 21, 22, **23**, **24**, 24, 25, **26**, 55, 60, **74**
TB	32	18·5	4·75	**4**, 5, 6, 6, 7, 7, 8, 11, **12**, 12, 12, **13**, 13, 16, 16, 18, **20**, 20, 21, **22**, 22, 22, 23, 25, 27, **32**, 41, 42, **52**, 61, 96, **101**
CA	29	25	6·21	0, 5, 6, 9, 11, 13, 15, 16, 19, 23, 23, 23, 24, 24, 25, 32, 35, 37, 37, 39, 39, 42, 42, 44, 81, 103, 105, *S*, *S*
CB	28	21·5	5·03	4, 4, 6, 10, 12, 12, 15, 15, 15, 17, 17, 18, 20, 21, 22, 22, 23, 25, 26, 28, 29, 30, 32, 37, 39, 40, 57, *S*
43 *TA*	36	15·5	3·92	2, **5**, 5, 6, **7**, 7, 7, 8, 9, **10**, 10, 10, 11, **12**, 12, 12, 13, **15**, 16, 16, 17, **19**, 19, **20**, 20, 23, 23, **24**, 25, 26, 26, 29, **31**, **31**, 33, 53
TB	34	24	5·88	7, 8, **10**, 12, 12, **14**, **15**, 15, 17, **19**, 20, 21, 22, 22, 22, **24**, **24**, 24, **25**, 25, **26**, 27, 27, 28, 32, 34, **35**, 43, 44, 45, 46, **47**, 63, **65**
CA	43	21	4·93	0, 2, 4, 6, 6, 7, 8, 8, 9, 9, 10, 10, 11, 11, 14, 15, 15, 18, 20, 20, 20, 21, 21, 21, 22, 23, 23, 24, 24, 24, 24, 24, 24, 34, 35, 39, 39, 41, 119, 128, *S*, *S*, *S*
CB	8	—	6·62	15, 17, 24, 25, 36, 36, 37, *S*
44 *TA*	37	17	4·43	**3**, 3, 4, 6, 6, 6, 6, **8**, **8**, 8, 9, 10, 11, **12**, **13**, 13, 15, 16, 17, 18, 18, 18, **20**, **20**, 23, **24**, 24, 26, **28**, 28, **33**, 38, 39, **40**, 55, *S*, *S*
TB	36	15	3·83	**4**, **7**, 7, **9**, **9**, **10**, **10**, 10, 10, **11**, 11, 11, **12**, 12, 12, 12, 13, 14, 16, 16, **17**, 17, **18**, 19, 20, 20, **21**, 21, 21, **22**, 23, **25**, 27, 29, **35**, 37
CA	29	20	5·27	3, 5, 7, 8, 9, 9, 10, 11, 12, 14, 16, 17, 19, 19, 20, 22, 23, 24, 24, 28, 33, 39, 48, 61, 62, 64, 87, *S*, *S*
CB	30	11	3·30	5, 5, 5, 6, 6, 6, 6, 7, 7, 7, 9, 9, 10, 10, 10, 12, 12, 13, 13, 14, 14, 15, 15, 16, 17, 17, 28, 43, 92, *S*

Table 3 (*continued*)

Generations		No. of rats	Median	Mean class	No. of errors made by each rat
45	*TA*	36	15	3·72	0, 2, 3, 4, **5**, 5, 6, **7**, 7, **8**, **8**, **9**, **9**, 10, 12, 12, 13, **15**, 15, 15, 16, 17, 19, 20, 21, 22, 22, 23, 24, 25, 26, **29**, **30**, 33, **35**, **45**
	TB	37	17	4·13	**3**, **5**, 6, **7**, 7, 8, **9**, **9**, 10, 10, 11, 11, 11, 11, 14, 15, **16**, 16, **17**, 17, 17, **18**, **19**, 19, 21, 21, **23**, 23, 23, **24**, 24, **25**, 28, 34, **35**, **41**, *S*
	CA	33	17	4·18	0, 1, 3, 4, 6, 7, 8, 9, 10, 10, 11, 11, 11, 15, 17, 17, 17, 17, 18, 21, 23, 23, 24, 24, 25, 25, 25, 25, 26, 29, 32, 53, 68
	CB	38	17	4·26	0, 0, 4, 4, 6, 8, 9, 10, 10, 10, 11, 11, 11, 12, 13, 14, 15, 16, 17, 17, 18, 18, 20, 22, 22, 23, 23, 23, 24, 25, 28, 29, 31, 33, 39, 40, 48, 52
46	*TA*	34	18	4·73	2, 4, 8, **9**, **10**, **10**, **13**, **13**, **15**, 15, **17**, 17, 17, 17, 17, **18**, 18, 18, 19, **20**, 21, **23**, 24, 24, **26**, 26, **27**, 29, 33, 36, 37, 41, 42, **64**
	TB	38	26	6·34	11, 12, 13, **19**, 19, 20, 20, 21, 21, **22**, 22, 22, **23**, **23**, 23, **24**, 24, 24, **26**, **26**, 27, 27, **28**, 28, 29, **31**, 32, **33**, **35**, **36**, 37, 40, 41, **46**, 47, 75, *S*, *S*
	CA	40	22·5	5·37	0, 3, 6, 6, 7, 13, 14, 15, 15, 16, 17, 17, 18, 19, 20, 20, 21, 21, 22, 22, 23, 23, 23, 23, 24, 25, 25, 26, 26, 27, 29, 34, 36, 41, 41, 45, 53, 80, 87, 110
	CB	29	22	4·90	2, 3, 4, 4, 9, 10, 11, 12, 15, 16, 16, 18, 18, 18, 22, 23, 24, 24, 27, 27, 28, 28, 31, 32, 34, 37, 40, 52, *S*
47	*TA*	34	20	4·79	**1**, **2**, 3, **5**, **6**, **6**, 6, **7**, **11**, 12, 12, 14, **16**, **17**, **18**, 18, **20**, 20, 21, **22**, 24, 24, **25**, 25, 26, 30, 31, 32, 33, 33, 37, 45, 50, 150
	TB	34	17·5	4·94	3, 6, **7**, **7**, 9, 10, **11**, 11, **12**, 12, 12, 13, 14, **15**, 15, 15, **16**, **19**, **20**, **22**, 25, **27**, 27, **28**, 29, 29, 32, **33**, **34**, 40, 46, 81, 100, *S*
	CA	39	20	4·72	1, 5, 5, 5, 6, 8, 9, 10, 10, 11, 13, 15, 15, 16, 17, 17, 17, 18, 19, 20, 20, 21, 22, 22, 25, 26, 28, 28, 29, 29, 30, 30, 31, 32, 34, 34, 35, 37, 46
	CB	34	26	5·44	3, 3, 4, 5, 6, 6, 7, 10, 11, 13, 13, 14, 17, 20, 21, 22, 25, 28, 29, 30, 30, 30, 31, 32, 33, 33, 34, 35, 36, 50, 59, 60, 70, *S*
48	*TA*	35	21	4·88	**5**, 8, **9**, 10, **11**, 12, 12, **14**, 14, 14, **15**, 15, 17, 17, 19, **20**, 20, **21**, **22**, 22, 22, 23, **24**, 24, 25, 27, 28, 29, **30**, **31**, 32, 36, 36, 58, 65
	TB	35	22	5·31	6, 7, 7, **8**, 8, **9**, **11**, **12**, 13, 14, 15, **17**, 17, 19, **21**, **21**, **22**, 22, 23, **26**, 26, **29**, 31, 31, **32**, 32, 33, 33, 33, 35, **36**, 37, **41**, **42**, 44
	CA	35	15	3·83	5, 5, 5, 6, 6, 7, 8, 8, 8, 8, 9, 9, 11, 12, 12, 12, 15, 15, 15, 15, 16, 16, 18, 19, 19, 23, 23, 24, 25, 29, 30, 32, 39, 49, 50
	CB	25	20	4·32	7, 8, 8, 9, 9, 10, 12, 13, 14, 16, 17, 18, 20, 20, 21, 24, 26, 27, 27, 29, 31, 53, 91, 110, 111
49	*TA*	35	20	5·09	**5**, **9**, 9, 9, 10, **11**, 11, **12**, 12, 13, **15**, 15, **16**, 16, 18, 19, **20**, 20, 20, **21**, **23**, **24**, 24, 24, 27, **28**, **31**, 31, 34, 38, 42, **47**, **48**, **62**, *S*
	TB	35	20	4·86	**7**, **10**, 10, **11**, 11, 12, 12, 13, 13, **15**, 15, **17**, 17, 18, **19**, 19, 19, **20**, **23**, 23, 23, **24**, **26**, 26, 26, 26, **27**, 28, **29**, **29**, **31**, 31, **38**, 39, **41**
	CA	34	17·5	4·62	2, 3, 7, 9, 11, 13, 13, 13, 13, 13, 14, 14, 15, 15, 16, 16, 17, 18, 19, 22, 22, 23, 24, 25, 25, 25, 25, 27, 29, 29, 30, 52, 87, *S*
	CB	35	22	5·54	2, 7, 8, 9, 11, 12, 13, 13, 15, 15, 15, 16, 18, 19, 19, 20, 21, 22, 23, 24, 25, 28, 30, 32, 33, 36, 38, 39, 40, 50, 56, 58, 76, 113, *S*
50	*TA*	50	19·5	4·74	2, 5, 6, 7, 8, 8, 8, 9, 9, 10, 10, 11, 12, 13, 15, 16, 16, 17, 17, 17, 17, 18, 19, 19, 19, 20, 20, 20, 20, 21, 22, 23, 23, 25, 25, 25, 28, 29, 30, 30, 30, 30, 30, 32, 36, 36, 37, 40, 47, *S*
	TB	50	18	4·62	7, 7, 7, 7, 8, 8, 8, 9, 9, 9, 9, 10, 11, 12, 12, 12, 12, 13, 13, 14, 14, 14, 16, 17, 18, 18, 18, 19, 19, 20, 21, 22, 22, 22, 23, 23, 25, 27, 28, 32, 33, 33, 34, 36, 39, 50, 58, 68, 71, *S*
	CA	50	18·5	4·78	4, 4, 4, 5, 5, 5, 6, 7, 7, 7, 8, 8, 9, 10, 11, 12, 13, 14, 15, 15, 16, 17, 17, 18, 18, 19, 20, 20, 21, 21, 22, 24, 25, 27, 27, 28, 31, 32, 33, 34, 34, 37, 37, 39, 42, 48, 56, 138, *S*, *S*
	CB	50	13·5	3·82	2, 4, 4, 5, 6, 6, 6, 7, 7, 7, 7, 8, 8, 9, 9, 9, 9, 10, 10, 10, 10, 10, 11, 12, 12, 12, 15, 16, 17, 19, 19, 20, 22, 22, 23, 23, 23, 24, 24, 25, 25, 25, 26, 27, 27, 28, 31, 31, 32, 33, 34

last six generations were of the same order as those of generations 13–16. Throughout the whole experiment the parallelism between the performances of the trained and control lines was remarkable; over the last few generations the controls were generally even superior to the trained line.

Table 4. *Summary of the results of the fifty generations in groups of four generations*

Generations	No. of rats		Mean class			% in class 1		% in class 10	
	T	*C*	*T*	*C*	Ratio: *T*/*C*	*T*	*C*	*T*	*C*
1–4	64	54	7·89	7·89	1·00	0·00	0·00	25·00	27·78
5–8	116	91	7·34	6·49	1·13	3·45	4·40	23·28	10·99
9–12	105	177	6·23	6·33	0·98	6·67	3·95	10·48	11·86
13–16	187	148	4·41	5·01	0·88	15·51	10·81	6·42	5·41
17–20	230	200	5·07	5·29	0·96	11·30	11·50	4·35	14·00
21–24	197	183	4·46	4·75	0·94	18·78	16·39	6·09	6·01
25–28	189	197	4·11	4·66	0·88	25·40	7·61	4·23	5·58
29–32	160	150	5·00	5·03	0·99	11·88	8·67	4·38	8·00
33–36	180	199	6·12	6·02	1·02	3·89	3·52	6·67	12·06
37–40	199	183	5·83	6·05	0·96	4·52	1·64	3·01	10·93
41–44	280	227	4·50	5·03	0·89	7·14	6·22	3·21	8·44
45–48	283	273	4·87	4·71	1·03	16·61	20·51	1·76	1·83
49–50	170	169	4·80	4·65	1·03	14·71	21·89	1·77	3·55

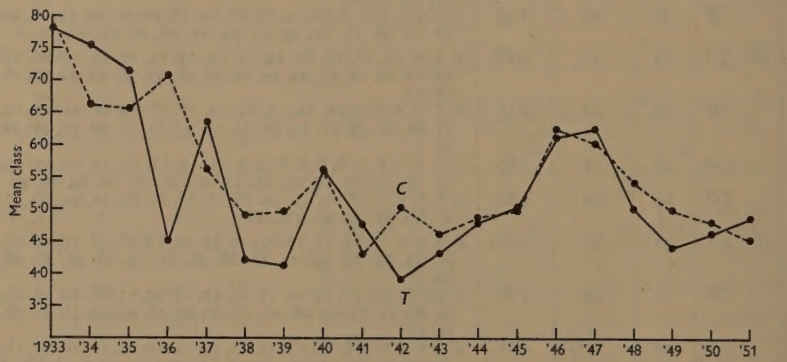

Fig. 2. Continuous line, line *T*; broken line, line *C*. The mean classes are those of all rats which began training in the year referred to. The first point, 1933, includes some rats which began training late in 1932; the last point, 1951, includes some rats which began training early in 1952.

It is unfortunate that McDougall did not publish full details of the performances of his rats; his reports give only the arithmetic mean of the scores in each generation and the scores of the best and worst rats. But although an accurate comparison of the rate, and extent, of changes in learning in the two experiments cannot be made, it is clear that the improvement which characterized our first twenty-eight generations closely parallels that of McDougall's thirty-two generations and it seems probable that the same factor, or factors, operated in the two experiments. But

McDougall's claim that the improvement was due to Lamarckian inheritance is plainly invalidated first, by the performance of our control line and secondly, by the fact that, in our experiment, the improvement was not maintained in later generations.

DISCUSSION OF RESULTS

What is the explanation of the observed changes in the rate of learning? Selection can be ruled out. McDougall found that improvement was continued when he deliberately practised adverse selection and, in our experiment, it would be difficult to explain on any selection hypothesis, why, with a standardized system of training and mating, the direction of change in rate of learning should be periodically reversed. The parallel performance of the trained and control lines suggests that

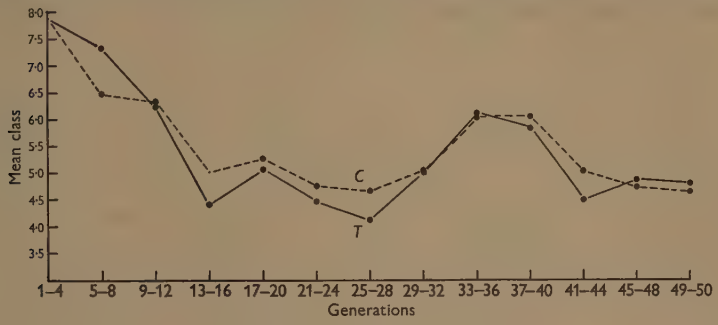

Fig. 3. This figure shows Table 4, column *Mean Class*, in the form of a graph. Continuous line, line *T*; broken line, line *C*.

the changes were related to factors, not necessarily having any genetic basis, which influenced the rate of learning.

In our First Report we listed six such factors : (1) the severity of the punishment, (2) vigour, (3) intelligence (ability to learn by experience), (4) the strength of the right or left habit, (5) 'venturesomeness', (6) chance factors not causally related to the learning process at all. Subsequent analysis of the data has shown that yet another factor needs consideration and that the performance of the rats was influenced by the season of the year in which they were trained. The separate or additive effects of these seven factors could explain the great variation in the performances of individual rats and the differences between particular generations, but only the first two, i.e. severity of punishment and vigour, seem to offer any basis for an explanation of trends of improvement or decline extending over a number of successive generations.

(*a*) *Seasonal effect*

When the rats of the whole fifty generations of the two lines are grouped according to the month in which they commenced their training and the mean class is calculated for each of the 12 months, it is found that, starting in February, the

means increase regularly to reach a maximum value in July and then decrease regularly to a minimum value in November (Fig. 4, Table 5).

In order to find whether this seasonal factor had operated throughout the experiment, similar analyses were made after dividing the experiment into four 5-year periods. In the early months of the year the results were erratic, but from March–April onwards generally conformed to the pattern of the previous analysis and showed consistently that rats which commenced their training during the winter months of June, July and August were at a disadvantage by comparison with those which commenced training in November and December.*

This effect may have been due, in part, to our failure to maintain a constant temperature in the colony room and in the water in the tank. The only precautions taken were, that in winter, the room was heated and warmed water was used in the tank. In neither case, however, was the temperature raised to summer levels.

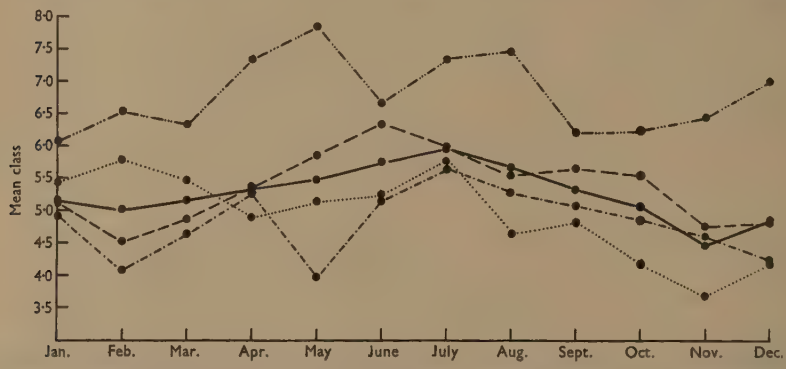

Fig. 4. The mean classes are those of the rats which began training in the month and period referred to. ——, 1932–52; —···, 1932–7; ...., 1938–42; – – –, 1943–7; –·–, 1948–52.

The lower water temperature during the winter may have had a slightly adverse effect on the performance of the rats, for Wever (1932) and Hack (1933) have found that the rat's incentive to escape from immersion varies with water temperature, being greatest at low temperatures. At lower temperatures (10–20° C.) the rats after being placed in the water swam strongly and directly to the landing platform. At temperatures from 30 to 40° C., Wever states that 'many of the animals did not head for the goal immediately but spent some time in casual exploration'. Hack describes the rats at 37·5° C. as showing an 'inquisitive attitude' with frequent reversals and re-entries into the blind alleys of the maze. This latter type of behaviour, we believe, facilitated learning in our experiment, but we are disinclined to attribute seasonal variation in performance to differences in water temperature; for the greatest range in temperature would not have exceeded 12–22° C. and the

* The main divergences from the pattern are the low values of the class mean in the May group, 1948–52, and the June group, 1932–37. In both groups the number of rats was small—considerably less than half the average number for the other months.

Table 5. *The rats of lines T and C combined classified according to the month in which they began their training*

		Month in which training began											
		Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
1932–7	No. of rats	67	40	97	36	39	15	45	83	84	38	49	66
	Mean class	6·09	6·52	6·33	7·33	7·84	6·67	7·34	7·45	6·20	6·24	6·43	7·00
1938–42	No. of rats	21	69	103	154	32	60	114	94	115	116	147	127
	Mean class	5·43	5·79	5·46	4·89	5·12	5·23	5·79	4·63	4·81	4·16	3·69	4·17
1943–7	No. of rats	90	70	151	103	80	109	90	139	115	182	41	99
	Mean class	5·13	4·51	4·89	5·35	5·86	6·36	5·98	5·55	5·65	5·56	4·75	4·82
1948–52	No. of rats	227	93	177	73	54	95	146	109	112	146	247	82
	Mean class	4·92	4·09	4·61	5·26	3·96	5·15	5·61	5·29	5·09	4·85	4·52	4·21
1932–52	No. of rats	405	272	528	366	205	279	395	425	396	482	484	374
	Mean class	5·18	5·01	5·17	5·33	5·48	5·72	5·95	5·67	5·32	5·06	4·48	4·84

maximal temperatures would have occurred in summer, not in November, which was the period of minimum scores. But in any case, seasonal effects cannot possibly account for trends, lasting over a period of some years, and it is primarily with these that we are here concerned.

As will be shown later, we do attach great importance to the effects on learning, of differences in behaviour in the tank, but we believe that these differences were largely the expression of variations in the health and vigour of the rats.

(*b*) *Severity of punishment*

In his preliminary experiments, McDougall found that the strength of shock had considerable effect on the rate of learning. Thus if the severity of punishment tended to wax and wane over long periods the performance of the rats would show corresponding trends. We are satisfied that this effect did not operate in our experiment. The importance of a standardized punishment was recognized from the beginning and the precautions taken to ensure this (see First Report) have been maintained. The electrical installations, as shown by periodical tests, have remained in perfect order.

(*c*) *Vigour*

McDougall, Crew and ourselves have all noted that less vigorous rats tended to learn more quickly. Crew attributed this to their receiving more severe punishment. We, however, agree with McDougall that it was mainly a result of their slower and more hesitant progress through the water which gave them more time to perceive the situation.

McDougall, while admitting the relationship, was inclined to minimize its effect. He wrote (1938, p. 374): 'very great vigour and liveliness is a little unfavourable to quick learning and a somewhat diminished or less-than-average vigour is probably slightly favourable'. Crew, having noted that poorly developed, feeble rats learnt quickly, suggested that 'as a general rule, the more vigorous the rat the higher the score may be expected to be'. In our First Report we produced evidence to support this view. Fifteen rats of the 3rd–5th generations which, before the 10th day of training and before learning, had been noted on the training cards as 'weak' or 'undersized' had an average score well below the general average of these generations. There was also evidence from generation 17 which was severely debilitated by a mite infestation. After this had been controlled and the diet changed there was an extraordinary improvement in the health of the colony and a striking change for the worse in training performances; forty-five rats trained prior to the eradication of the mites had a class mean of 3·29; thirty-five rats born afterwards had a class mean of 6·40. But in the following generations, although the rats remained in a good condition, there was a return to low training scores and we were then inclined to accept McDougall's conclusion that variation in health and vigour could not explain the fluctuations in the rate of learning.

Further experience led us to revise this opinion. During the course of the experiment, the general health and fertility of the rats varied considerably. At irregular intervals the colony went into a decline extending over several generations

and then, for no apparent reason, regained its health and vigour. McDougall evidently had the same experience for he refers to 'waves of decline of vigour'. Greenman & Duhring (1931), working with a selected group of rats at the Wistar Institute, report large weight fluctuations over a succession of generations so that changes in the general physique of the albino rat may occur, even in colonies maintained in the most favourable environment. The occurrence of such changes in our colony, coupled with the facts set out above, justified a detailed analysis of possible relationships between health and training scores.

As the need had not been foreseen, proper records of the health of the rats were not kept and the data available are therefore few. We have, however, fairly complete fertility records, and as fertility is correlated with health these have provided us with an indirect measure of health. Since all the members of a single generation were mated at the one time, they were all therefore given an equal chance of reproducing; and it is justifiable to use, as an index of fertility, the number of fertile rats in each generation, expressed as a percentage of the number mated.*

The rats were weaned at 26 days and commenced their training on the 28th day. The great majority (about 80%) learnt during the first fortnight of training, i.e. between the ages of 31 and 42 days, when their general health would be determined, in large measure, by the nursing capacity and therefore the general health of the mother.

Thus if rate of learning were influenced by the health of the rats one would expect a correlation between *fertility, used as a measure of health, of one generation, and the training scores of the next.*

The first analysis was made in respect of generations 1–40, i.e. for the period prior to the splitting into sublines. The coefficient of correlation, for the trained line was +0·40 and for the control line +0·42. Both values are significant at the 1% level, and fertility is therefore shown to be positively correlated with high training scores.

The analysis was then extended to include the whole fifty generations, treating the trained rats and the controls each as a single population. For the trained line there was again a positive correlation significant at the 1% level. For the control line the correlation was positive but was not significant.

When the control sublines of generations 41–50 were analysed separately, it was found that there was no significant correlation between fertility and training scores in either subline, but that in subline *B*, such correlation as occurred, was negative. This latter fact was not wholly unexpected for until the 48th generation it had been obvious at the time of training that subline *B* was atypical in that, while fertility was fairly high, the rats were undersized and poor in health. Their inferiority was indicated by their small litters. In every generation from 41 to 48, the average litter

* In the trained line our records show, as fertile, only those rats whose offspring were taken into training. When fertility was high, some first litters were discarded without their parentage being recorded and the fertility index would thus give an underestimate of the true level of fertility. This situation did not often arise, and, in fact, the value of the index for the trained line was not significantly different from its value for the controls, where all first litters were required, either for breeding or training.

size in subline *B* was smaller than in subline *A*. For the eight generations combined the average for *B* was 7·3, for *A* 9·3.

While there are grounds for regarding control subline *B* as atypical, the occurrence of a negative correlation between fertility and scores is disconcerting. It does not, however, discredit the highly significant correlation established for the first forty generations of the control line and for the whole fifty generations of the trained line.

The possibility that there might be a correlation between fertility and rate of learning was considered by both Crew and McDougall. Crew pointed out that if there were a positive correlation between fertility and quickness, i.e. the reverse of the one established above, it could explain the progressive improvement of McDougall's rats. McDougall had been fully aware of the significance of the point made by Crew, but such evidence as he collected, supports our conclusion. In his water-maze experiments, several attempts to isolate a superior stock, by breeding from selected quick learners, failed on each occasion because of the infertility of the superior rats. He had the same experience in his first selection experiment with quick learners in the tank.

We have established a positive correlation between the fertility of one generation and the scores of the next but it is difficult to believe that there could be any direct causal relationship between them. They must have been connected by a third factor. The basic premise of the foregoing analysis is that this third factor was the general health and vigour of the rats. If it be accepted that healthy vigorous rats are more fertile than less vigorous ones, then the above correlation indicates that the more vigorous the rat the higher its expected training score.

Tryon (1929, 1932) has suggested that the reverse relationship may hold for maze-learning. By breeding rats selectively, according to their ability on a maze, he developed a strain of 'brights' and a strain of 'dulls'. Over a number of generations he found that both lines showed a progressive improvement in learning ability. Tryon was inclined to attribute this improvement to increased vigour of the rats. Were this so, it would not necessarily conflict with our conclusion on the influence of vigour on learning in the tank. Krechevsky (1932) has analysed the performances of Tryon's two strains, and has concluded that the difference between them was related specifically to maze learning. The 'brights' learned quickly because they used 'spatial hypotheses'. The 'dulls' used 'visual hypotheses'. Drew (1939) has pointed out that this, while penalizing them on a maze, would have favoured them in a light-discrimination test, and that Tryon's 'dulls' would probably have been quick learners in the tank. The two learning situations were so different that vigour could have been favourable in one, and a handicap in the other.

The training scores of individual rats were necessarily influenced by a variety of factors and all of these, no doubt, played some part in causing the changes in the average rate of learning which occurred during the course of the experiment. We believe, however, that the major changes, that is the changes involving a progressive improvement or decline extending over several generations, were due primarily to changes in the general level of health in the rat colony. The cause of

these fluctuations in health of the colony is quite unknown. It may be that they result from infection, and that recovery from a decline involves a selection process, in which the enfeebled strain is eliminated through diminished fertility.

In retrospect it seems that we have been less successful than Crew in standardizing the factors that cause variation in the rate of learning. This has, however, had the positive advantage that it has enabled us to obtain the effect of a progressive improvement in learning rate that McDougall found. McDougall attributed it to the operation of Lamarckian inheritance. Our own results forbid this interpretation for the effect is not sustained, and is displayed in equal measure by the controls.

SUMMARY

This is the final report of an experiment of 20 years' duration, in which we have repeated, in its essentials, the well-known experiment of William McDougall purporting to reveal a Lamarckian inheritance of the effects of training on rats. The test is one involving light discrimination, and McDougall recorded a steady improvement in the rate of learning on a succession of 32 generations; but he omitted to check the results against a properly conducted control.

Our experiment confirms McDougall to the extent that we too have obtained long duration trends of improvement in learning-rate (Figs. 2, 3); but we find that the effect is not sustained, and that it is, moreover, shown also by a control experiment, using animals of untrained ancestry. This forbids a Lamarckian interpretation.

Statistical analysis of the data indicates that the 'condition' of the rat markedly affects its speed of learning, and that progressive changes in learning-rate, over a succession of generations, are in reality correlated with the health of the laboratory colony, which is subject to periods of decline and recovery.

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STUDIES ON THE TRANSPORT OF NEUROSECRETORY MATERIAL IN *CALLIPHORA ERYTHROCEPHALA* BY MEANS OF LIGATURING EXPERIMENTS

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(With Plates 7–9)

1. THE PROBLEM

In an earlier investigation on the function of the neurosecretory brain cells and the corpus cardiacum of the adult blow-fly *Calliphora erythrocephala* (E. Thomsen, 1952) I came to the conclusion that the medial neurosecretory cells (m.n.c.) must be regarded as the overall controlling centre of the endocrine system in this fly. It was found that the corpus cardiacum was not able to function unless it had been activated by the m.n.c., and in all probability this also applied to the corpus allatum. This was tested on the developmental stage of the ovaries.

In these experiments it was not possible to decide by which mechanism the m.n.c. influence the corpus cardiacum and the corpus allatum. However, when the corpus cardiacum-allatum complex was removed from a young fly and transferred to the abdomen of the same individual it was found to be able to function in 50% of the cases. In this experiment it is evident that the activation of the corpus cardiacum-allatum by the m.n.c. must have taken place via the blood, i.e. it was hormonal, for no nervous connexion existed between the brain and the transplanted organs. Naturally this experiment does not tell anything about the way by which the hormone from the m.n.c. reaches the corpus cardiacum-allatum in normal individuals. The hormone might either be given off to the blood, or it might travel inside the axons of the neurosecretory cells (n.c.).

That such a transport takes place in some insects has been suggested by B. & E. Scharrer (1944) and supported by Cazal (1948). This ingenious idea has since been corroborated by other investigators (Stutinsky, 1952; B. Scharrer, 1952*a*, *b*; Hanström, 1953; M. Thomsen, 1954*a*; Arvy, Bounhiol & Gabe, 1953), but at the time when my above-mentioned experiments were performed few facts were known. Our own attempts to demonstrate stainable material in the axons of the m.n.c. in their course inside the brain of normal *Calliphora* gave negative results except in a single case. However, in some *Hymenoptera* beautifully stained axons were observed (M. Thomsen, 1954*a*), and this observation prompted me to try to show experimentally that a transport of secretory material takes place in the axons of the n.c. of *Calliphora*.

In the last few years a good deal of evidence for the transport of secretion in the axons of n.c. in different animals has been brought forward. Much of this evidence is morphological, but the problem has also been tackled experimentally by several investigators. I shall return to these experiments in the discussion.

2. LIGATION OF THE CARDIAC-RECURRENT NERVE

In *Calliphora* the nerves from the neurosecretory cells of the protocerebrum, the nervi corporis cardiaci I and II, immediately after leaving the brain fuse with the nervus recurrens to form a single nerve, which we might call the cardiac-recurrent nerve. This nerve runs through the narrow neck to the corpus cardiacum + ganglion hypocerebrale situated in the foremost part of the thorax.

If there is a flow of secretory material in the axons of the n.c. it should be possible to block this flow. This might be done either by simply cutting the nerve or by ligating it. When the nerve is cut there is a possibility that part of the secretory material might diffuse out into the blood. In the ligated nerve, however, most of the material ought to remain inside the nerve, provided that the nerve does not break.

A series of experiments was therefore started in which the cardiac-recurrent nerve was ligated. As the nerve lies below the aorta, the part of the aorta above the nerve had to be removed. Fortunately the cutting of the aorta does not do any harm to the animal. The ligature was made with very fine silk thread taken from the cocoon of the silk-moth. In order to get the thread under the nerve, the nerve was lifted a little by means of a tiny piece of hair.

Ligation of the nerve was performed upon a total of 100 flies. Of these flies fifty-six were 6–8 hr. old (reared at 25° C.) at the time of the operation; the remaining forty-four flies were 22–35 hr. old.

The flies were kept from 3 to 7 days (at 25° C.), after the operation and fed on meat, sugar and water. The mortality was heavy, only forty-eight flies surviving. All the flies with ligated nerves were first examined alive under Ringer's solution with a binocular.

In eighteen of these flies the knot had disappeared or was lying freely in the coagulated blood; this of course means that the nerve was broken. In sixteen flies the nerve was broken in front of the knot, and in two flies behind the knot. In twelve cases only the nerve was found to be unbroken. In the cases in which the nerve was broken it is of course impossible to say when this took place—it might even have happened during the final inspection, because as a rule the knot is hidden by a clot of coagulated blood which must be removed in order to examine the nerve and the knot.

In the majority, i.e. in twenty-seven flies, the proximal part of the nerve was clearly swollen and had a pronounced bluish tinge. The colour of the proximal part of the nerve very much resembled that of the m.n.c. In thirteen other flies the proximal end of the nerve was swollen and contained small bluish globules or lumps. In some of the flies a few axons were plainly visible as bluish stripes. In five cases nothing special was seen, and in three cases (from the first experiments) I have no notes concerning the appearance of the nerve in the living fly.

It is worthy of note that not in a single case was the distal end of the nerve (that is, the part of the nerve behind the knot) found to be swollen or to have a bluish tinge or to contain bluish lumps.

In my opinion it is most likely that the bluish tinge and the bluish lumps in the proximal portion of the nerve are secretory material, the flow of which has been blocked by the ligature.

In the beginning of the investigation I thought that it would be impossible to picture the piling up of the secretory material in the living ligated nerve. So I tried to show it in sectioned nerves.

Owing to the hard cuticle of *Calliphora* the nerve could not be sectioned *in situ*, but had to be removed. As, however, the nerve is rather thin, about 30μ in diameter only, it was too difficult to fix and section it isolated, and it was therefore necessary to dissect the brain out together with the nerve.

Twenty-two brains were dissected. The brain and the nerve were fixed either in Bouin's or Helly's fluid, cut in serial sections of 10μ each and stained with Gomori's chrome-haematoxylin-phloxin. Seven cases were unsuccessful. Of the remaining fifteen preparations seven did not present anything special, but the eight preparations left showed something of interest. The two best of these are pictured.

Fly no. S3, f5, was kept for 3 days after the operation. In this fly the nerve was broken. The inspection of the living fly with the binocular revealed that the end of the nerve was swollen and had a bluish tinge, and a few bluish globules were visible. In the section of the nerve (Pl. 7, fig. 1) some distinct axons are seen, which owing to their content of material stain bright red. At intervals they are thickened, and their ends are clearly swollen, forming small balls. The red material is homogenous.

In a neighbouring section (Pl. 7, fig. 2) two axons are seen to terminate in bulbs.

Fly no. S12, f3, was kept for 5 days after the ligature was made. In this fly the nerve was unbroken, and in the living fly the part of the nerve in front of the knot was clearly swollen, bluish, and contained several blue lumps. The part of the nerve behind the knot was not swollen, it did not have the bluish tinge and did not contain any lumps.

In this case I succeeded in dissecting out the brain with the nerve and the knot *in situ*, but unfortunately the knot disappeared during the sectioning. Pl. 7, fig. 3 shows a sagittal section through this nerve. Two kinds of material are clearly visible, big red lumps and a dark blue material which in some cases formed moniliform strings. The big lumps of material are found at some distance in front of the knot.

It is interesting that in the cases in which bluish lumps were seen in the *living* nerve, the sections showed big stained aggregations of material.

As far as I know, in all the insects hitherto studied the n.c. and the secretory material in the axons of these cells were found to stain blue with Gomori's stain. So at first I found it rather puzzling that some of the material in the nerve of *Calliphora* was red. But by now we have found that some of the m.n.c. in *Calliphora* may also stain bright red with the phloxin of the Gomori's stain, and blue and red

n.c. are sometimes found in the same section. In this connexion it was most interesting to learn that Bliss & Welsh (1952) found that the secretory material of the X-organ in crabs when stained with Gomori's stain assumes either a homogenous acidophilic or granular basophilic form.

Thus it seems that in *Calliphora* the secretory material may either appear in a form which stains blue with the haematoxylin of the Gomori's stain or in a form which stains red with the phloxin.

Even if the stained preparations described above give a fairly good evidence for a transport of secretory material in the axons of the n.c. of *Calliphora* it would be more impressive if the secretory material could be made visible in the *living* ligated nerve. It turned out that darkfield illumination was very suitable for this purpose. I also tried phase-contrast microscopy, but the result was not quite as good.

In all, thirteen of the ligated nerves were observed with darkfield illumination, and the best of these were photographed.

Pl. 7, fig. 4 shows the proximal part of the living ligated nerve from a female which lived for 3 days after the operation. The nerve was found to be broken, but already the inspection with the binocular revealed that the proximal end of the nerve contained some bluish lumps. In the photograph it is seen that the end of the nerve is swollen and lumps of secretory material are clearly visible.

Pl. 7, fig. 5 shows the proximal end of the nerve from a fly which lived for 5 days after the ligation of the nerve. Here again the nerve was broken. Under the binocular the proximal end of the nerve looked bluish white and farther up the nerve some bluish lumps occurred. In the photograph the accumulated secretory material is white and so are the two or three axons with definite swellings of secretory material.

In Pl. 8, fig. 6, is seen the ligated nerve from a fly which lived for 3 days after the operation. The knot is in place and the nerve is unbroken. It is seen at once that the part of the nerve in front of the knot has an appearance which is quite different from the part behind the knot; it is thicker and has another colour. The white colour indicates the piling up of secretory material caused by the constriction of the nerve. In one of the axons some distinct lumps are seen. (Unfortunately this part of the nerve was torn somewhat during the dissection.)

The part of the nerve just behind the knot is rather thin; this is due to the constriction; and no white colour is seen in it. In the living fly it had a faint yellowish colour. The preparation looked very beautiful when alive, the difference in the tinge of the two parts of the nerve being much more pronounced than in the picture.

The results of the ligation experiments, as seen in the stained and the living nerves, leave little doubt that normally a flow of secretory material takes place from the neurosecretory cells of the brain through the axons into the corpus cardiacum, and that this flow is obstructed by the ligature.

3. THE NORMAL (UNLIGATED) CARDIAC-RECURRENT NERVE

A comparison of the ligated cardiac-recurrent nerve with the normal (unligated) cardiac-recurrent nerve proved to be helpful in the interpretation of the experimental results. *A priori* it was not expected that secretory material would be discernible in the living unligated nerve, but it turned out that in nearly all cardiac-recurrent nerves examined with darkfield illumination such material could be observed.

Photographs of two such nerves are shown in Pl. 8, figs. 7 and 8. In fig. 7 the cardiac-recurrent nerve divides into two branches before entering the corpus cardiacum; this is a normal phenomenon. In general only a few axons of the cardiac-recurrent nerve contain secretory material, which is seen to form moniliform strings. It is most interesting that the moniliform strings in these living nerves very much resemble the axons of n.c. in stained sections.

In the normal unligated nerve I have never seen big lumps of the kind found in the ligated nerves.

The nervi oesophagei, which pass from the corpus cardiacum + ganglion hypocerebrale to the intestine, were likewise found to contain some moniliform axons, strikingly similar to the axons containing secretory material in the normal cardiac-recurrent nerve (Pl. 9, fig. 9).

Axons of the same appearance were also discovered in the living nervi corporis allati, which go from the corpus cardiacum to the unpaired corpus allatum. Unfortunately I have not yet succeeded in getting a photograph of these nerves.

Quite recently secretory material has been stated to occur in the axons of the nervi corporis allati of *Gryllus domesticus*, *Blatta orientalis* and *Periplaneta americana* (Stutinsky, 1952), of *Bombyx mori* (Arvy *et al.* 1953), and of *Synagris* and *Eumenes* (Hymenoptera) (M. Thomsen, 1954*a*, *b*). In all these insects the secretory material was demonstrated in sections by means of the chrome-haematoxylin-phloxin stain, which stains the secretory material a dark blue.

As the nervi corporis allati probably branch off from the nervi corporis cardiaci it is not surprising that they include nerve fibres containing secretory material. As to the axons with secretory material observed in the nervi oesophagei, it is at present not possible to say where the corresponding cell bodies are located.

4. THE NORMAL AND THE LIGATED ABDOMINAL NERVE

The occurrence of secretory material in the normal unligated cardiac-recurrent nerve, the nervi oesophagei, and the nervi corporis allati, made it important to study another living nerve as a control, and for this purpose the abdominal nerve was chosen.

Excised pieces of the living abdominal nerve from a number of flies have been studied with darkfield illumination, but in no case could axons be detected having an appearance similar to that of the axons in the nerves just mentioned.

In Pl. 9, fig. 10 is shown an excised piece of a living abdominal nerve. The axons

of this nerve are seen as delicate filaments. These filaments are not of quite the same thickness throughout, but have a slightly varying diameter.

As another control the abdominal nerve was ligated in a number of females. Fortunately this nerve is rather easy to get at from the ventral side.

In all, twenty females were so treated and six of these flies died before autopsy. of the surviving fourteen flies five were dissected on the third day and nine on the fourth day after the ligature was made. In three flies only was the nerve found to be unbroken. In these cases there was a pronounced swelling of the nerve in front of the knot, and a minor though still conspicuous swelling behind the knot. In the darkfield the part of the nerve in front of the knot was whitish, not bluish, and in no case were bluish lumps found.

A ligated abdominal nerve is seen in Pl. 9, fig. 11. The white lines are tracheae. The nerve is conspicuously swollen just in front of the knot, and somewhat swollen behind the knot. The tinge of the nerve before the knot was not bluish and no bluish lumps were found. The white spots behind the knot are probably ends of tracheae.

In the eleven flies in which the nerve was broken both ends of the nerve were swollen, the proximal end was however always thicker than the distal one. The proximal end was never found to have a bluish tinge or to contain bluish lumps.

5. DISCUSSION

My investigations show two points which may claim some interest. First it was found that neurosecretory material could be seen in the living axons by means of darkfield illumination. With the same method secretory material can also be demonstrated in the living neurosecretory cells (M. Thomsen, 1954*a*). In a short paper Passano (1951) stated that he had seen discrete droplets of secretory material in the single living axons of the X-organ-sinus-gland nerve of crabs by means of the phase-contrast microscope.*

Secondly the experiments support the view that secretory material, which is built up in the n.c., moves through the axons of these cells into the corpus cardiacum and the corpus allatum. Very likely this is the way in which the n.c. normally control the two organs mentioned. It is presumed that only when the nerve has been cut does this secretory material pass into the blood, and the control of the two organs is effected through this medium.

Since previous experiments have shown (E. Thomsen, 1952) that the 7-day-old corpus cardiacum, i.e. the activated corpus cardiacum, may replace the m.n.c. in their effect on egg-development, it is very likely that the activation of the corpus cardiacum in this case is synonymous with a storage of secretory material from the n.c. in the corpus cardiacum, from where it is given off into the blood. The idea that the corpus cardiacum might be regarded as an organ of storage was first suggested by B. Scharrer (1951) for *Leucophaea*.

* Quite recently, Carlisle (1953) made the important communication that he had been able to observe the passage of secretory material along the axons of the X-organ connective of *Dromia* and *Lysmata* (Crustacea). This observation was also made by means of the phase-contrast microscope.

The result of the ligating experiments in *Calliphora* is very well in line with the findings of other authors concerning the same problem.

When I started the experiments reported above I only knew the experiments of Hild (1951), who demonstrated that severance of the stalk of the pituitary of the frog caused an accumulation of stainable material proximal to the site of interruption of the axons. A corresponding result was obtained by Stutinsky (1951) in frogs and rats, and by E. Scharrer & Wittenstein (1952) in dogs. The beautiful experiments of B. Scharrer (1952*a*,*b*) on *Leucophaea* clearly showed that severance of one of the two nerves from the n.c. of the brain to the corpora cardiaca is followed by a storage of secretory material in the proximal stump of the severed nerve, and an eventual depletion of stainable material in the corpus cardiacum of the same side. Finally, Passano (1951), working on crabs, stated that severance of the nerve from the X-organ to the sinus-gland resulted in an accumulation of secretory material in the proximal end of the nerve.

There is therefore now strong evidence for the idea, already advanced by B. & E. Scharrer in 1944, that neurosecretory material produced in the pericaryon of n.c. migrates down the axons of these cells.

It is most interesting and gratifying that this idea is in very good accordance with the results of Weiss (1944) and Weiss & Hiscoe (1948) on the regeneration of nerve fibres in rats. These authors came to the conclusion that growth in the sense of production of new protoplasm occurs solely in the nucleated part of the nerve cell. Even in the mature fibre, which has reached a stationary condition, the axoplasm is maintained in constant proximo-distal motion. If this continuous proximo-distal motion of the axoplasm is a general characteristic of neurons, it seems that the transport of visible secretory material in the axons of n.c. could be regarded as a special case of a general process.

SUMMARY

Ligation of the cardiac-recurrent nerve in adult females of *Calliphora* resulted in an accumulation of secretory material in the proximal part of the nerve. The secretory material was demonstrated partly in stained sections, partly in living nerves by means of darkfield illumination.

By the latter method moniliform bluish white nerve fibres could be seen in the living normal (i.e. unligated) cardiac-recurrent nerve. In all probability this appearance of the nerve fibres is due to their content of neurosecretory material. Some axons of the nervi oesophagei and the nervi corporis allati had a similar appearance. No such fibres could be observed in the living abdominal nerve, and ligation of this nerve did not result in an accumulation of secretory material.

I should like to express my gratitude to the Carlsberg Foundation which has supported this work with a grant. A preliminary paper has been published recently (E. Thomsen 1954).

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EXPLANATION OF PLATES

Plate 7

Fig. 1. Sagittal section of proximal stump of a cardiac-recurrent nerve, 3 days after ligation. *a*, axons containing secretory material; *b*, bulb-shaped ending of axon. ×300.

Fig. 2. Same preparation as in fig. 1, neighbouring section. Two axons are seen to terminate in bulbs. ×300.

Fig. 3. Sagittal section of the proximal part of a cardiac-recurrent nerve, 5 days after ligation. *a*, moniliform axon stained dark blue; *b*, red-stained bulb, four of which are seen in the section; *p*, position of knot, which has disappeared. ×300.

Fig. 4. Proximal end of living cardiac-recurrent nerve, 3 days after ligation. *b*, lump of secretory material; *p*, position of knot. Darkfield illumination, ×130.

Fig. 5. Proximal end of living cardiac-recurrent nerve, 5 days after ligation. *a*, axon with swellings of secretory material; *p*, position of knot; *s*, accumulation of secretory material. Darkfield illumination, × 130.

PLATE 8

Fig. 6. Living cardiac-recurrent nerve, 3 days after ligation. The knot is *in situ*. *a*, axon with swellings of secretory material; *d*, distal part of nerve; *e*, ends of silk thread; *k*, knot; *p*, proximal part of nerve. Darkfield illumination, × 190.

Fig. 7. Living normal (unligated) cardiac-recurrent nerve with moniliform axons containing secretory material; *c*, corpus cardiacum. Darkfield illumination, × 190.

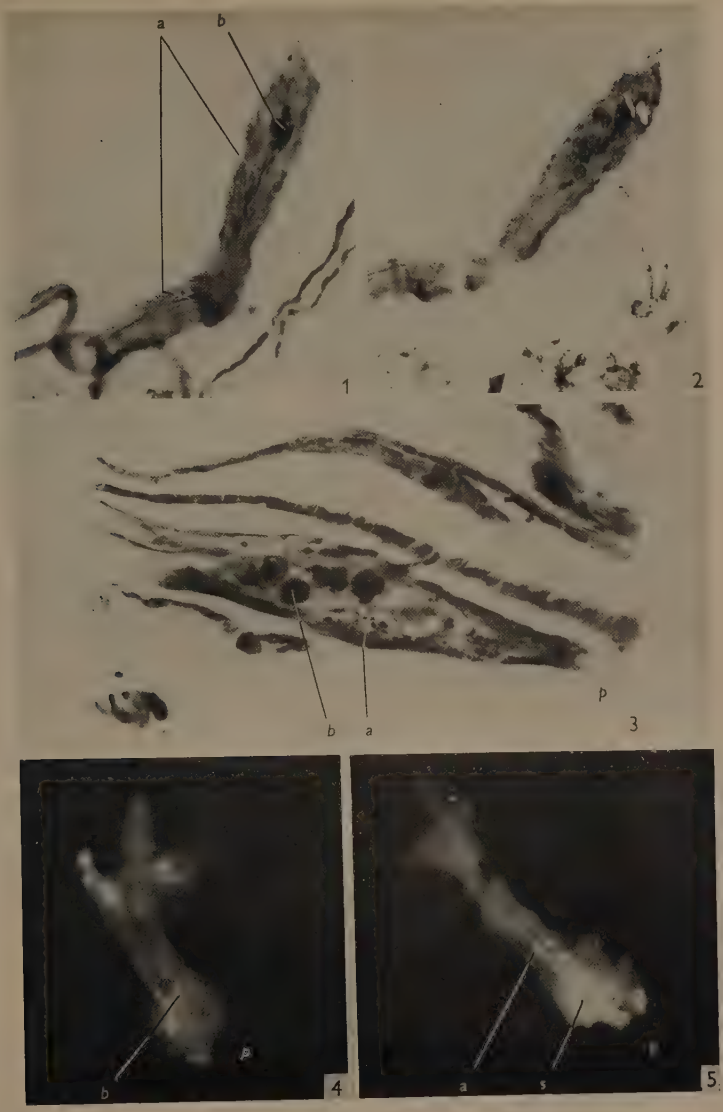
Fig. 8. Living normal (unligated) cardiac-recurrent nerve. *a*, axon with secretory material; *c*, corpus cardiacum; *tr*, tracheae. Darkfield illumination, × 190.

PLATE 9

Fig. 9. Living nervus oesophageus with an axon containing secretory material. *c*, corpus cardiacum. Darkfield illumination, × 190.

Fig. 10. Living normal (unligated) abdominal nerve. *tr*, tracheae; cf. text. Darkfield illumination, × 190.

Fig. 11. Living abdominal nerve, 3 days after ligation. The knot is *in situ*. *d*, distal part of nerve; *e*, ends of silk thread; *k*, knot; *p*, proximal part of nerve; *tr*, tracheae; *x*, probable end of trachea. Darkfield illumination, × 65.



THOMSEN—TRANSPORT OF NEUROSECRETORY MATERIAL IN *C. ERYTHROCEPHALA*

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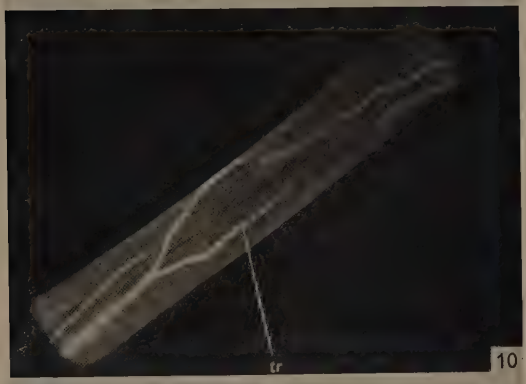

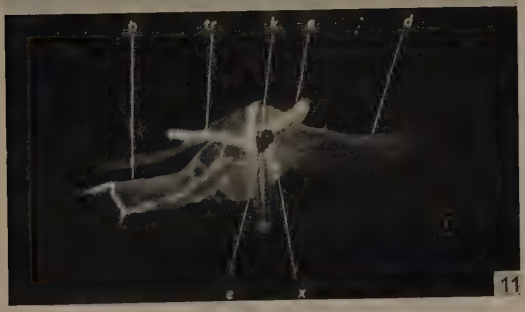

THOMSEN—TRANSPORT OF NEUROSECRETORY MATERIAL IN *C. ERYTHROCEPHALA*

WATER BALANCE IN THE TICK *ORNITHODOROS MOUBATA* MURRAY, WITH PARTICULAR REFERENCE TO THE INFLUENCE OF CARBON DIOXIDE ON THE UPTAKE AND LOSS OF WATER

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INTRODUCTION

Lees (1946, 1947) has shown that in many species of ticks water may be absorbed from air so much unsaturated with water vapour as to preclude the possibility of the water entering the tick in response to an osmotic gradient between the body fluids and the surrounding air; the gradient is in the opposite direction. He showed that water uptake under these circumstances was under the control of active forces within the cells of the tick itself and that the cells concerned were probably those of the epidermis.

Ticks are partially protected from losing water at humidities below their equilibrium humidity—that humidity at which water is neither gained nor lost by the tick—by the presence of a thin layer of wax in their epicuticle. In the Argasidae the wax layer, which is protected by a cement layer in this group, is composed of a relatively high melting-point wax and confers a high degree of impermeability upon the cuticle. Other attributes which assist in the retention of water are an efficient mechanism for closing the spiracles (Browning, 1954) and the ability of the tick to restrict, in some manner, the outward passage of water. There is evidence that the epidermal cells are concerned in this also.

In many ticks the uptake of water does not suffer interference if the spiracles are covered, and in *Ixodes ricinus* this treatment does not alter the rate of transpiration in low humidities. In these cases, at least, water exchanges must occur through the general cuticle surface. When similar experiments are carried out with *Ornithodoros moubata*, however, the ticks become asphyxiated.

Many insects are known to be caused to keep their spiracles open when exposed to an atmosphere containing 5% carbon dioxide (Mellanby, 1934; Wigglesworth, 1935). Mellanby (1935) showed that this was also true for engorged nymphs of *O. moubata*, but Lees (1946) could not confirm it in unfed females of *Ixodes ricinus*.

The work presented below is an attempt to resolve these differences in the results obtained with carbon dioxide on ticks, and to inquire further into the activity of ticks in absorbing water and in restricting water loss.

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MATERIALS AND METHODS

A stock of *Ornithodoros moubata* was built up from females kindly supplied by Major H. S. Leeson, of the Department of Entomology, London School of Hygiene and Tropical Medicine, to whom grateful acknowledgement is made.

O. moubata does not feed as a larva but moults directly to the first-stage nymph. Thereafter nymphs moult only after feeding and may remain for months between feeds in a healthy condition if kept at room temperature in Petri dishes having in the bottom a piece of filter-paper which is moistened occasionally.

In most experiments second-stage nymphs were used, but other stages were found convenient on occasion and these are specified in the text.

Ticks were fastened to small wire hooks by a tiny drop of wax on their mid-dorsum. From these they were suspended, during experiments, in humidity chambers, which were either 2 × 1 in. specimen tubes containing one tick, or 500 ml. conical flasks containing up to ten ticks in cases where gas mixtures were used.

Gas mixtures were prepared over water, bubbling the gas into an inverted measuring cylinder. From this the mixture was drawn out through concentrated sulphuric acid into the flasks.

Lees (1946) showed that the equilibrium humidity for unfed nymphs of *O. moubata* lay between 80 and 90% relative humidity. In these experiments a relative humidity of 95% was considered to be above the equilibrium humidity of the ticks, and this condition is referred to as *humid air* in the text. The only other humidity condition used was air standing over concentrated sulphuric acid and this is referred to in the text as *dry air*.

Humidities were controlled with solutions of sulphuric acid placed in the tubes or flasks. The temperature was kept constant at 25 ± 1° C. Weighings were made to the nearest 0·02 mg. on a torsion balance.

RESULTS

(1) *Experiments on water loss and water absorption in air*

Most of the results obtained in these experiments confirm those obtained by Lees (1946, 1947) or simply extend his observations. They will therefore be stated very briefly and the necessary data presented.

Engorged ticks before moulting do not absorb water from humid air, even after considerable desiccation in dry air. Their weight remains fairly constant or may fall slightly over a few days.

Engorged ticks lose water more rapidly in dry air than do unfed ticks of a comparable surface area. For example, five second-stage nymphs each lost an average of 0·02 mg. per day in dry air before feeding. They were then fed, left for 24 hr. on blotting-paper at room conditions to complete excretion and loss of coxal gland fluid, and then replaced in dry air. The average rate of water loss during the following 3 days was 0·14 mg. per tick per day. The surface areas of the ticks were of course identical before and after feeding.

From these experiments it is clear that after feeding some of the tick's ability to regulate its water balance is lost, and this must be due to the failure of the active mechanism controlling water exchange, since the spiracle-closing mechanisms are still operative (see below), and there is no reason to suppose that the epicuticle undergoes any change.

Throughout the moulting cycle the rate of water loss from engorged ticks remains unchanged (see Fig. 6B), although the endocuticle is eroded almost completely during this time. The epicuticle, however, remains intact until the moment of ecdysis.

Newly moulted ticks, suspended in humid air, do not increase in weight. If they are first desiccated for some days in dry air and then transferred to humid air they absorb water rapidly and their weight may rise higher than it was before desiccation began. The rate of water uptake under these conditions is much more rapid than the rate of loss in dry air (Fig. 1). In first-instar nymphs the rate of loss of water in dry air was found to average approximately 0·01 mg. per tick per day, whilst the rate of water uptake from humid air averaged 0·03 mg. per day.

Ticks which were suspended for periods of 8 or 9 days in dry air alternating with periods of 6 or 7 days in humid air alternately lost and gained water for some time (Fig. 1). However, the capacity to absorb water from humid air gradually diminished as the ticks aged, but not in any very uniform way. One tick (designated by open circles in Fig. 1) lost water almost continuously from the 41st day to the 107th day even though, during this time, it had spent four periods of 7 days each at 95% R.H. On the 107th day, on being reintroduced into humid air, it began absorbing water and took up over 30% of its body weight in 4 days. After this it again became very erratic in regard to water absorption and finally died after 150 days.

Fig. 1 shows that the sequence of weight loss and gain was very regular for the first 30–40 days, and that thereafter it became quite irregular. This is probably due to the gradual depletion of the food reserves of the ticks as they age (Lees, 1948). The source of energy for the sudden bursts of activity after prolonged periods of quiescence remains obscure.

Blocking the spiracles of unfed nymphs of *O. moubata* resulted in their failing to absorb water from humid air (Lees, 1946; and see Fig. 2). It was also found to result in a greatly increased rate of water loss in dry air (Fig. 3). Blocking only one spiracle had no such influence on water loss or gain. It would seem that blocking the spiracles leads to asphyxiation, as Lees suggested, but whether as the result of oxygen lack or carbon dioxide accumulation is not known.

(2) *Experiments with ticks suspended in mixtures of carbon dioxide and air*

Unfed nymphs of *O. moubata* were suspended over concentrated sulphuric acid in flasks and the carbon dioxide concentration of the atmosphere in the flasks was increased progressively day by day. It was found that the daily rate of water loss remained constant until the concentration of carbon dioxide reached between 30 and 45%, when the rate increased markedly (Fig. 4). The ticks in any one flask

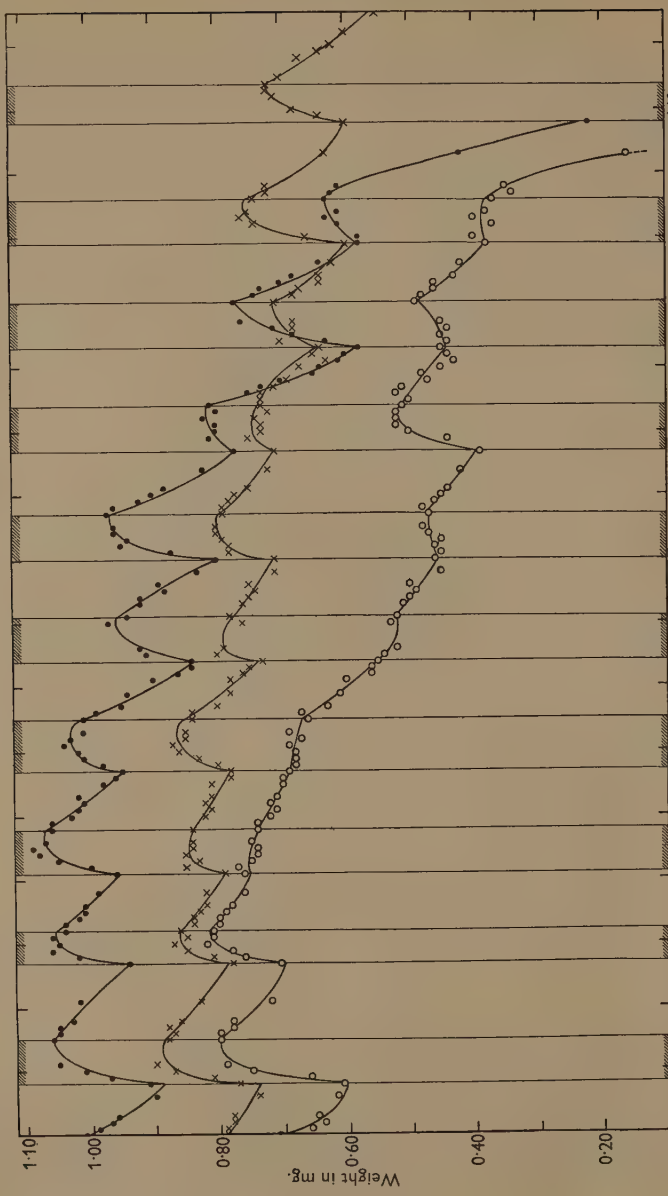

Fig. 1. Changes in weight of three second-instar nymphs, beginning on the day after moulting. Hatching indicates periods during which the relative humidity was 95%. The remainder of the time was spent at 0% R.H.

(usually five) always behaved similarly, but different batches began to lose water rapidly at different carbon dioxide concentrations.

An experiment with ticks walking freely in dry flasks filled with different carbon dioxide-air mixtures showed that the ticks became anaesthetized at about the same concentrations as caused the increase in the rate of water loss (Table 1).

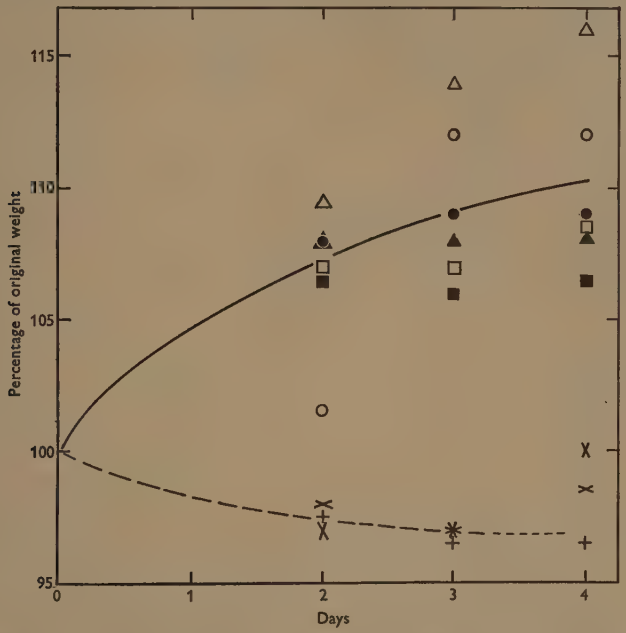

Fig. 2. Changes in weight of unfed second-instar nymphs kept at 95% R.H. after having been desiccated for some time. Solid symbols: untreated ticks; open symbols: ticks with one spiracle blocked; crosses: ticks with both spiracles blocked.

Table 1. *The influence of different concentrations of carbon dioxide on the behaviour of unfed second-stage nymphs. Five ticks in each flask*

CO_2 concentration	Behaviour after 1 hr.	Behaviour after 16 hr.
50%	All quiescent	Three on backs; two upright, immobile
35%	One on back, legs moving slowly; four walking very slowly	One walking very slowly; four immobile
20%	All moving slowly	All walking apparently normally

That the effect of carbon dioxide on water loss is one of anaesthesia rather than asphyxia is supported by the effect of increased nitrogen concentrations on water loss. Under these conditions no increase in the rate of transpiration was observed until the nitrogen concentration of the atmosphere reached about 98% (the

remaining 2% being oxygen), at which point the ticks were observed to become greatly agitated and the rate of water loss rose markedly (Fig. 5).

Carbon dioxide has a similar influence on water absorption. Desiccated unfed nymphs absorbed water normally when suspended in humid atmospheres con-

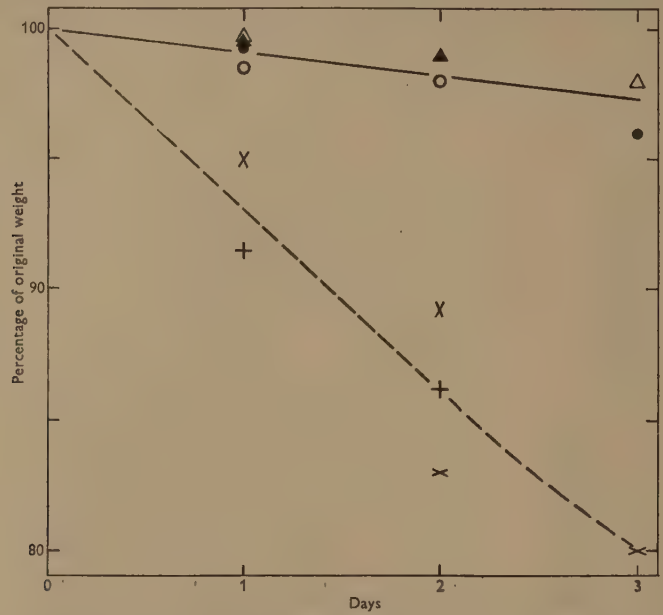

Fig. 3. Changes in weight of unfed second-instar nymphs kept at 0% R.H. Symbols as in Fig. 2.

Table 2. *Numbers of unfed second-stage nymphs whose weights either increased, decreased or remained unchanged during 24 hr. at 95% R.H. and in various concentrations of* CO_2

Weight ...	Increased	Decreased	Unchanged
CO_2 concentration			
0%	4	—	—
15%	4	—	—
20%	1	1	—
30%	1	—	1
35%	1	—	2
50%	—	2	4

taining increased concentrations of carbon dioxide up to the point where the gas began to anaesthetize them. In higher concentrations the ticks remained constant in weight or may even have lost water (Table 2), although the atmosphere was above their equilibrium humidity.

These experiments suggest that the effect of carbon dioxide on water exchange is

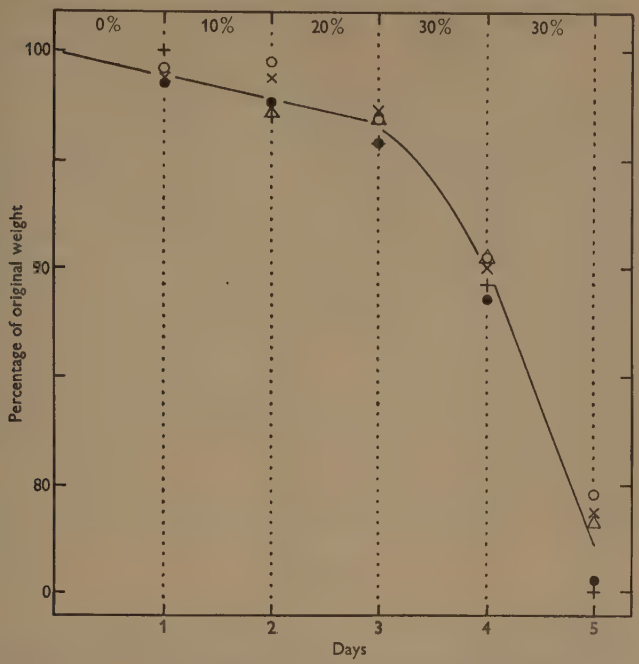

Fig. 4. Changes in weight of five unfed second-instar nymphs kept at 0% R.H. in air and air-CO_2 mixtures. The concentration of CO_2 during each 24 hr. period is shown at the top of the graph.

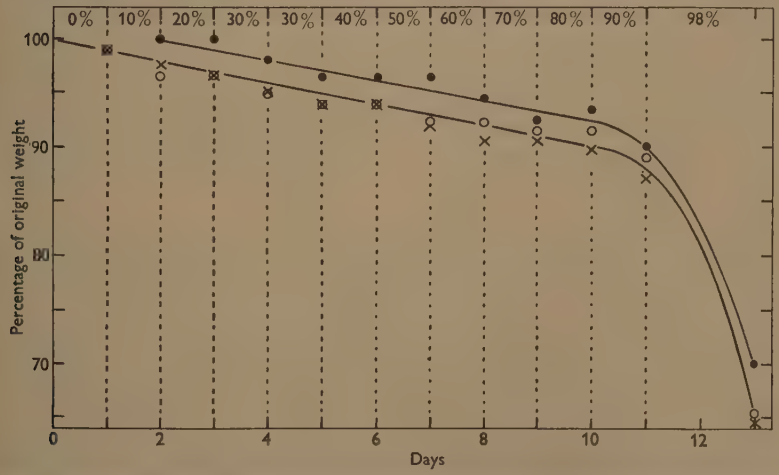

Fig. 5. Changes in weight of five unfed second-instar nymphs in dry air and dry air-N_2 mixtures. The concentration of N_2 during each 24 hr. period is shown at the top of the graph.

produced by inactivation of the epidermal cells (if these are indeed the active organs regulating water exchange) rather than simply by causing the spiracles to remain open.

When engorged ticks, suspended over concentrated sulphuric acid, were treated with increasing concentrations of carbon dioxide in air it was found that the rate of water loss increased markedly in 5 % carbon dioxide compared with the rate in air. Further increase in the concentration of carbon dioxide did not cause any further increase in the rate of loss of water (Fig. 6A), although the ticks became anaesthetized in 35 % carbon dioxide. Even in 95 % carbon dioxide the rate remained fairly constant, although the ticks appeared to become asphyxiated and some of them did not survive the treatment.

The effect of carbon dioxide observed here agrees with that found by Mellanby (1935), and his explanation that 5 % carbon dioxide causes the ticks to keep their spiracles open would seem correct.

Repeated experiments with 5 % carbon dioxide on unfed ticks gave results which indicated a slightly greater rate of water loss than occurs in air, but the results were not statistically significant.

In the experiment with engorged ticks no signs of moulting could be observed at the end of the experiment, whereas the controls moulted the day after the experiment ended.

DISCUSSION

Both the ability of ticks to absorb water from humid air and that part of their resistance to water loss in dry air which is not due to structural devices have been shown to be inhibited simultaneously by carbon dioxide anaesthesia. Both these attributes are also lost in the engorged tick and in the dead tick. Any injury such as abrasion of the cuticle (Lees, 1947) and slight burning produces both a marked increase in the rate of water loss and an inability to absorb water. It seems probable that the same mechanism is operative in both cases.

The fact that both processes are inhibited by high concentrations of carbon dioxide may mean that both are under the indirect control of some agency, perhaps the central nervous system, on which the carbon dioxide acts, for it has been shown that carbon dioxide anaesthesia inhibits not only the control of water exchange but also the moulting process and the main motor responses, both of which are under nervous control.

The difference in the behaviour of engorged and unfed ticks in response to 5 % carbon dioxide is probably more apparent than real. If the epidermal cells are in fact responsible for the active control of water loss, and if 5 % carbon dioxide does cause unfed ticks to keep their spiracles open, it is perhaps to be expected that this should not result in a markedly increased rate of transpiration, for the atrium of the spiracles and the tracheae are surrounded by a layer of epidermal cells. As these cells would also probably be active in absorbing water and preventing its escape the limiting factor in determining the rate at which water was lost through the spiracles might well be the rate at which water could escape into the tracheal system from the surrounding cells. In the case of the general epidermal cells this

has been shown to be rather slow, and the same may also be true of the cells surrounding the tracheae.

In engorged ticks, however, the situation is quite different.

Here the water-absorbing and water-retaining powers are not functioning, and water should be able to diffuse much more rapidly into the tracheae to replace that lost through the spiracles. Opening the spiracles in this case would be expected to lead to the marked increase in the rate of transpiration which was observed.

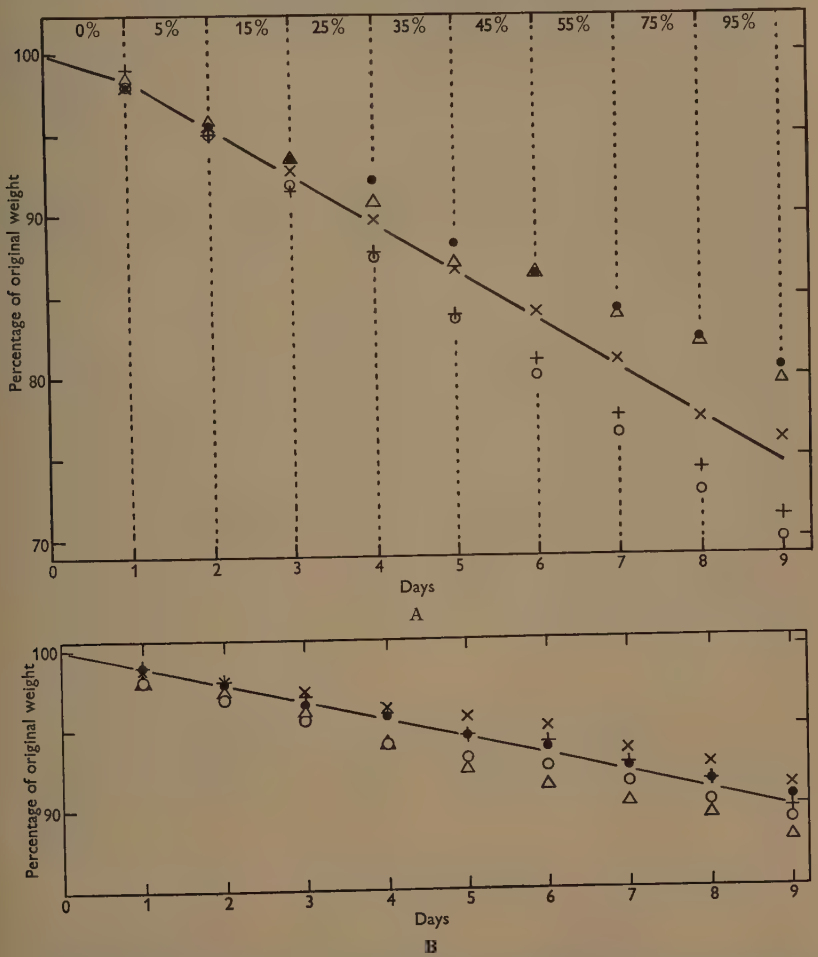

Fig. 6. A: changes in weight of five engorged second-instar nymphs in dry air and in dry air-CO_2 mixtures. The concentration of CO_2 during each 24 hr. period is shown at the top of the graph. B: changes in weight of five similar ticks to those in A, during the same period in dry air.

SUMMARY

1. A study has been made of the exchanges of water between the atmosphere and the tick *Ornithodoros moubata*.

2. Unfed nymphs are able to abstract water from moist air (95 % R.H.) and to restrict their rate of water loss in dry air.

3. This ability is lost (*a*) in atmospheres containing 30–45 % CO_2; (*b*) in atmospheres containing more than 90 % N_2; (*c*) immediately after the tick is fed; (*d*) gradually after the tick has been starved for some five months.

4. It has been shown that the effect of high (30–45 %) concentrations of CO_2 is mainly upon the activity of the epidermal cells, possibly mediated through the central nervous system. The concentration required to cause opening of the spiracles is only about 5 %.

It is a pleasure to acknowledge the hospitality and help I received from Prof. V. B. Wigglesworth, F.R.S., during my stay in his laboratory. Drs A. D. Lees and J. W. L. Beament were always ready to discuss problems and to them my thanks are also due.

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THE ORIENTATION OF ANTS

I. THE SUBSTITUTION OF STIMULI

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INTRODUCTION

The classic work of von Frisch (1923, 1946, 1948*a*, *b*, 1949, 1950, 1951) on communication in the honey-bee has shown that when a returned or prospective forager dances in the hive, the orientation of its dance pattern is related to the orientation of its foraging flight. If the dance is performed on the vertical surface of a comb, the foragers orientate relative to gravity at the same angle as they orientate their outward flight relative to the direction of the sun's rays. It is therefore difficult for the observer to avoid the conclusions of Thorpe (1949, 1950) that bees perform processes of symbolizing and map reading. The attribution of such abilities to the nervous system of insects was sufficiently unusual to stimulate a physiological investigation of the effect on an insect's orientation of interchanging the two stimuli of light and gravity.

For various reasons bees were unsuitable for a laboratory study, and as ants were already known to perform the light compass reaction (Brun, 1914; Santschi, 1911, 1923), and to be sensitive to gravity (Turner, 1907; Barnes, 1929, 1930*a*, *b*; Cornetz, 1914), these insects were selected for experimental subjects. Ants show no behaviour resembling the dance of the bee. The species used were *Myrmica ruginodis* (Nyl.) and *M. laevinodis* (Nyl.), which are very similar both in form and behaviour.

A series of experiments was carried out in which an ant was presented successively with two orientatory stimuli of different types. In the first series light and gravity were used as the interchangeable stimuli: the results of these experiments proved encouraging, and two further series were done; in one, plane-polarized light was interchanged with unpolarized light; and in the other, gravity was interchanged with plane-polarized light. These three series are described in different sections of this paper, and in a final discussion an attempt is made to correlate all the results and to consider the behaviour of the honey-bee in relation to them.

SERIES A. THE INTERCHANGE OF LIGHT AND GRAVITY

Apparatus and methods

When an ant is placed on an horizontal surface in a dark-room, and a beam of light is shone across the surface, the ant at first wanders short distances in apparently random directions. If the ant is stimulated by a light blow upon the substratum, or by a gentle touch on the abdomen with a glass rod, it will frequently start running

in a straight line, and will continue in the same direction for up to 20 cm. This behaviour will be called the 'escape reaction'. During the run the ant performs a light compass reaction relative to the light source. If the ant is on a vertical surface in the dark (or in dim red light) it orientates its track at a constant angle to the vertical—a menotaxis in Kuhn's terminology (Kuhn, 1919). It is proposed to call such an orientation a 'force compass' reaction. The actual angle of orientation in both types of compass reaction varies both with different ants, and with the same ant on different runs. The direction taken does not seem to be influenced by the locus of the releasing stimulus.

The object of the experiments was to release the escape reaction in an ant, and to present it successively with the two orientatory stimuli light and gravity. This was achieved by allowing the ant to run on the flat surface of a rectangular board (45 × 25 cm.), which was pivoted along its short axis, and could be held with its surface either horizontal or vertical. When the board was vertical it was illuminated by dim, diffuse red light from a dark-room safe lamp: when it was horizontal it was illuminated by an horizontal beam of light from an Osram 100 V. 60 W. opal bulb, placed 4 m. away. The board was held in its horizontal position by an electromagnetic relay, which also operated the light circuit. The whole apparatus, which was set up in a dark-room, was painted a dull black. The surface of the board was covered with a flat sheet of white cartridge paper, lightly pencilled into 1 in. squares. A fresh sheet of paper was used for each ant.

In the experiments an ant was placed on the apparatus and allowed to wander at random for about 2 min., to become accustomed to the conditions. The escape reaction was then released and, after the ant had travelled in a straight line for at least 4 cm., the board was swung smoothly into its alternative position, thus interchanging the two stimuli. The track of the ant was plotted continually: this was done by making light pencil marks where an ant entered and left a square, the marks being made only when the ant was one square ahead. After each run the track was plotted in detail in pencil. It was found that this method of plotting did not apparently disturb the ant, and that it did not respond to the pencil marks in any noticeable way.

Individual ants were not marked, and could not be identified. They were selected from three colonies, each about one hundred strong; forty-eight individuals were used, and the method of selection was such that it is very improbable that more that six individuals were used more than once. On an average five complete recordings were made with each ant; individuals became hyperexcited after a longer series, and were then unsuitable for experiment. For each individual the experimental conditions were kept constant, but for different individuals the direction of the light was changed.

Results

There was no significant difference between the results obtained when light was the initial stimulus (board horizontal), or when gravity was used first (board vertical). The following account applies equally to both conditions.

When an ant was running on an horizontal surface, it made a constant angle with the light beam; when on a vertical surface it made a constant angle with the vertical. When the board was swung from one position to another, thus interchanging the stimuli, one of two possible responses occurred; either the ant stopped temporarily and wandered short distances at random, in which case the observation was discarded; or the ant smoothly changed direction and set off in another straight line, orientating by the second stimulus. It is with the track resulting from such smooth changes of direction that we are concerned here. In order to obtain the first hundred of these tracks 170 runs were made. The behaviour of the ant is, therefore, as follows: first the ant runs in a straight line in a particular direction, it then changes direction as a result of interchanging the stimuli, and after turning continues running in a straight line in a new direction.

The length of each part of the track, and the angles of orientation to the first and second stimulus were measured. If any part of the track was less than 3 cm. long the reading for that part of the track was discarded, as the accuracy of recording was not sufficient to justify recordings for such short distances. Sometimes two changes of direction occurred after swinging the board; if the ant had travelled more than 3 cm.

Table 1

Conditions of experiment	No. of ants used	No. of readings
With board initially horizontal and finally vertical	22	121
With board initially vertical and finally horizontal	26	120

before the second turn, then the final section of the track was discarded; if it had travelled less than 3 cm. before the second turn, then the second part of the track was discarded. The angles of orientation were measured clockwise from the line of action of the stimulus (away from the light, and vertically downwards, respectively): this imposes limits of 0–360° on the angles, with the extremes coincident.

It was noticed that when an ant approached within 2–3 cm. of the edge of the board, it often turned and ran parallel to the edge: tracks in which this occurred were discarded. Table 1 gives details of the number of readings taken.

The orientation angles for the two parts of the track were compared by plotting a graph of orientation angle to light against orientation angle to gravity. This is shown in Fig. 1 below.

The points on this graph appear to lie in a particular but complex pattern. This pattern would correspond with the four lines of the equations

$$y = x,$$
$$y = 360 - x,$$
$$y = 180 - x,$$

and

$$y = 180 + x.$$

Unfortunately no practical method exists for the simultaneous calculation of four different relationships, or for the closeness of correlation of the variables

involved. If, however, the four equations given above are assumed to be correct, then the situation can be represented by the diagram in Fig. 2. It can be prophesied that if an ant is orientating at a certain angle to the first stimulus it will orientate (within certain limits of accuracy) to the second stimulus at one of four angles, which can be deduced from the diagram.

Consideration of Fig. 1 would lead to the expectation that if the orientation angles were recorded only as the acute angles between the tracks and the stimuli, irrespective of actual direction, then the graph of the successive orientation angles

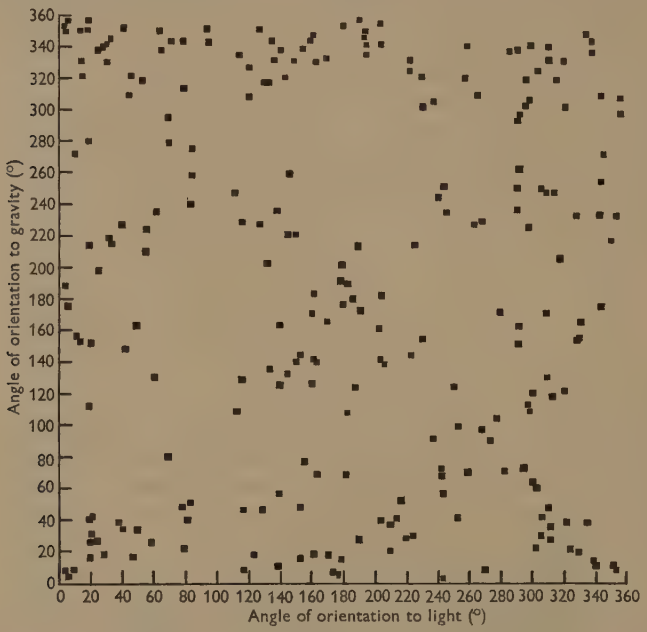

Fig. 1. *Experiment.* Interchanging light and gravity as orientatory stimuli. The results given are a combination of all readings obtained, including those from experiments when light was the initial stimulus, and those when gravity was used first. The angle of orientation to light is measured clockwise from the direction of the light. The angle of orientation to gravity is measured clockwise from the vertically downwards direction.

would show a single, simple relationship. Fig. 3 was therefore plotted, with the orientation angles measured in this way, a method which imposes limits of 0–90°.

Correlation coefficients were calculated for the figures used in Fig. 3; they are shown in Table 2.

Although this establishes that the two successive orientation angles are significantly correlated, it does not indicate that actual relationship. Calculation of a regression would not be helpful in this case, for both abscissa and ordinate are uncontrolled variables. Moreover, even if such a relationship were established for

Fig. 3 it would not be permissible to apply the result to Fig. 1, since it is not known whether the points falling about different lines in Fig. 1 contribute equally to the single line in Fig. 3. If, however, the regression in Fig. 3 is not the simple one of equation $x=y$, then it is difficult to understand how the symmetrical pattern in Fig. 1 could arise. For the purpose of further work the four equations given above are assumed to be correct.

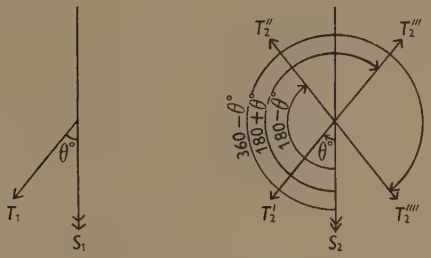

Fig. 2. S_1, first stimulus; S_2, second stimulus; T_1, first track; T_2', T_2'', T_2''', T_2'''', four possible second tracks.

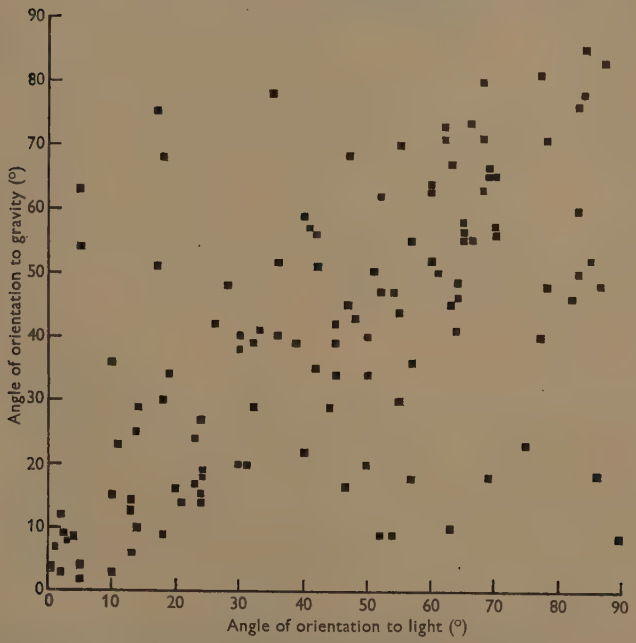

Fig. 3. *Experiment.* Interchanging light and gravity as orientatory stimuli. The results given are those obtained only when light was used as the initial stimulus. The graph obtained when gravity was used first was not significantly different from this. The angles of orientation in both cases are measured as the smallest angle between the track and the line of action of the stimulus, irrespective of actual directions. This angle must always be acute.

Discussion

The results described can be interpreted as showing that if an ant orientates successively to light and to gravity the two angles of orientation are related to each other in one of the four ways shown by the equations

$$\theta_1 = \theta_2,$$
$$\theta_1 = 360 - \theta_2,$$
$$\theta_1 = 180 - \theta_2,$$
$$\theta_1 = 180 + \theta_2,$$

where θ_1 = initial angle of orientation,

θ_2 = final angle of orientation.

Table 2

Conditions of experiment	Correlation coefficient	Degrees of freedom	Level of significance
With board initially horizontal and finally vertical	0·5869	119	0·1 %
With board initially vertical and finally horizontal	0·4506	118	0·1 %
The values of these correlation coefficients are not significantly different from each other.			

The results were examined to investigate what factors influence an ant to choose any one of the four possible tracks available after the change of stimulus. It was found that the following factors did *not* influence the choice:

(*a*) The size of the angle through which the ant has to turn to reach its new orientation.

It was observed that an ant never turned through more than 180°, but during the turn often passed a possible orientation.

(*b*) The original orientation of the ant.

(*c*) The orientation of the ant to the second stimulus immediately after interchange of the stimuli.

The angle between the lines of action of the stimuli (measured by drawing these lines on the paper and recording the angle between them as if they were in the same plane) did influence the choice slightly: if the angle between the stimuli was 0° or 180° there was a tendency for the ant to walk straight on after interchange of stimuli: if the stimuli were at 90° or 270° the successive orientations were not correlated with one another; ants tend to avoid orientating at 90° to both light and gravity, and this may have some relevance here. Apart from these conditions, however, the choice of the second orientation appeared to be random.

An attempt was made to do some control experiments, which involved omitting one of the stimuli when the board was in an otherwise appropriate position. When a stimulus was lacking an ant seldom maintained a straight track for more than about 3 cm., and seldom travelled continuously for any longer distance. It can be deduced that ants need an orientatory stimulus of some sort to travel in a straight line, although a hyperexcited ant may travel very fast in one direction independently

of the stimuli. However, with normal ants no experiments could be done to determine the effect of the apparatus itself on orientation. Since, to the ant, the apparatus consists of an isolated, flat surface in a dark-room, and since later experiments showed that under such conditions ants orientate either to light or gravity, it is thought that the apparatus itself did not provide landmarks or other guides which might influence orientation.

Individual ants showed a slight preference for one of the four possible relationships over the other three, but this effect was spread over the whole population of ants used, and consequently did not distort the complete picture.

The experiments were repeated using *Lasius niger* (L.). Sufficient results were obtained to show that the situation is the same for this species as for *Myrmica ruginodis*. The value of the correlation coefficient for the two angles of orientation was 0·356 with 64 degrees of freedom—significant at the 1% level. The accuracy of the relationship seems to be about $\pm 45°$ for *Lasius niger* compared with $\pm 30°$ for *Myrmica ruginodis*. The significance of this difference has not been investigated.

The discovery of a correlation between the successive orientations of an ant to light and to gravity leads one to wonder whether a similar result will be obtained for interchange of other stimuli. Some experiments to study this are now described.

SERIES B. THE INTERCHANGE OF LIGHT AND POLARIZED LIGHT

Apparatus and methods

It has previously been shown (Vowles, 1950) that ants orientate to the plane of vibration of a vertical (dorsal) beam of plane polarized light. Such a beam of light, circular in cross-section and with a diameter of 15 cm., was produced by shining the light from a 100 V. 100 W. opal bulb down a long, blackened tube, fitted with baffles to eliminate reflexions. Across the lower end of the tube was an horizontal sheet of polaroid mounted between two glass sheets on a turntable. The vertical beam of light passed down through the polaroid on to the floor of a dark-chamber. Rotation of the polaroid did not disturb the intensity or uniformity of the transmitted light.

An horizontal beam of light from a 100 V. 60 W. opal bulb was passed along a similar tube so that it intersected the vertical beam of polarized light on the floor of the chamber. The electrical circuit was arranged so that the observer could close a switch which interchanged the vertical, polarized light with the horizontal, unpolarized light.

The light intensity of the polarized light was 3·5 f.c., that of the other light 6·2 f.c.

In the region of intersection of the two light beams a circular disk of cartridge paper, lightly pencilled into 1 cm. squares, was glued to the floor of the chamber. The disk was 15 cm. in diameter, thus occupying the whole of the illuminated area. A fresh sheet of paper was used for each ant. The same method of plotting was used as in the previous experiments.

In the experiments an ant was placed on the illuminated surface (the apparatus was set up in a dark-room) and allowed to wander for about 2 min. to become accustomed to the conditions, being exposed for half this time to each of the two

light sources. Then, with either the horizontal or the vertical light on, the escape reaction was released. The track of the ant was plotted, and after it had travelled about 4 cm. the two lights were interchanged and the track again plotted. The ant was thus exposed successively to light and to polarized light. When the stimuli were interchanged the ant either stopped and wandered at random, or smoothly changed direction, as in the previous experiments; the tracks resulting from such smooth changes of direction were recorded. On an average four readings were taken with each ant, and the experimental conditions were kept constant for each individual: for different individuals the angle between the direction of the horizontal light and the plane of vibration of the vertical light was varied, and the order in which the stimuli were used was changed. Table 3 summarizes the number of readings.

Table 3

Conditions of experiment	No. of ants used	No. of runs	No. of readings
With horizontal light on initially	20	225	79
With vertical, polarized light on initially	20	190	80
Total	40	415	159

Results

The angles of orientation to the two stimuli were measured. A graph was then plotted of orientation angle to light against orientation angle to polarized light. No particular relationships could be seen on this graph, although the scatter of the points did not appear random. A second graph was then plotted of angle through which the ant turned (y) against angle between stimuli (x). The angle through which the ant turned was called positive when clockwise and negative when anticlockwise. The angle between the stimuli was the angle between the direction of the horizontal light and the plane of vibration of the vertical, polarized light, and was measured clockwise from the direction of the horizontal light. The results did not differ significantly with the order in which the two stimuli were used. All the results are plotted on Fig. 4.

Two relationships are obvious on this graph, the regression lines forming a diagonal (45°) cross, intersecting the x axis at approximately 90°. In the following statistical analysis the points lying at $x = 80°$, 90° and 100° were omitted owing to the difficulty of allotting these points to the correct line. The remaining points were allotted to the line to which they appeared closest, this allocation in fact giving the least residual sum of squares. The y values on the line of negative slope were multiplied by -1. This gave four lines all of positive slope. Analysis showed that these lines did not differ, either in slope or position. The results were therefore combined, and a common regression calculated. This was:

$$y = 0{\cdot}861x - 78{\cdot}2049.$$

This implies that when the stimuli are interchanged, the ant turns through an angle of approximately $78 - \theta°$ in either direction; where $\theta°$ is the smallest angle between the stimuli, measured clockwise or anticlockwise.

Discussion

The relationship disclosed above means that under constant experimental conditions the actual orientations of an ant to the two stimuli will in fact be correlated. In the experiments, however, the angle between the stimuli was different for different ants; since the actual relationship between the two orientations depends

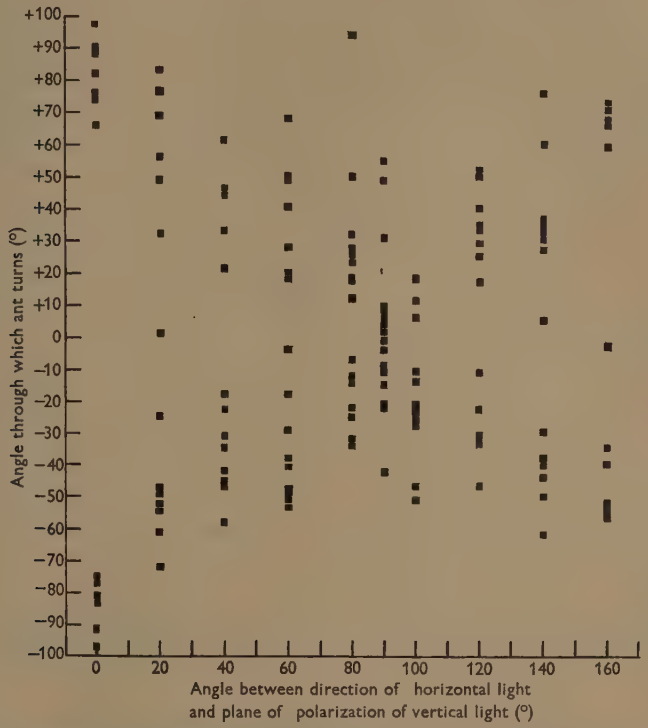

Fig. 4. *Experiment.* Interchanging horizontal light and vertical, polarized light as orientatory stimuli. The results given are a combination of all readings obtained, including those from experiments when the horizontal light was the initial stimulus and those when vertical, polarized light was used first. The angle between the plane of vibration of the polarized light and the direction of the horizontal light was measured clockwise from the direction of the horizontal light. The angle through which the ant turned is called positive if clockwise, negative if anticlockwise.

upon this angle, and since it is different for different values of this angle, one would not expect a graph of orientation angles for all ants to show any particular relationships. The results do show that successive orientations to light and polarized light are correlated in a complex way. The significance of this will be discussed later.

Under natural conditions the plane of vibration of the light from the blue sky overhead is at right angles to the plane of incidence of direct sunlight. The experiments show that under these conditions, when the stimuli are interchanged, an ant does not deviate very much from its actual heading. This may have some adaptive significance in the field, where clouds may temporarily obscure the sun.

SERIES C. THE INTERCHANGE OF POLARIZED LIGHT AND GRAVITY

The experiments already described for the interchange of light and gravity were repeated using plane polarized light. Except that the horizontal board was illuminated by a vertical beam of polarized light, the conditions were as before.

The behaviour of the ants was similar to that described for the other substitutions, although in this series the tracks were rather shorter. Again, on a certain number of occasions, the ant smoothly changed direction after the interchange of stimuli.

Table 4

Experiment	No. of ants used	No. of runs	No. of smooth changes of direction
Interchanging gravity and polarized light	24	231	119

With these results, however, no relationships could be detected either between successive orientations or between angles of turn. It is possible that no such relationships exist. It is equally possible, however, that the relationships exist but are obscured by their own inaccuracy. If, for example, there were four or more relationships involved, and since for these ants the accuracy of orientation to gravity is only $\pm 33°$, and to polarized light $\pm 26°$, it is possible that the total accuracy of relationships involving orientations to both stimuli might be as little as $\pm 59°$; points lying in bands of this width on either side of a regression line would undoubtedly overlap with points from other such regressions, thus preventing recognition of the two relationships. The experiments therefore neither support nor condemn the hypothesis that successive orientations to two types of stimuli are correlated.

GENERAL DISCUSSION

The experiments on the interchange of light and gravity, and of light and polarized light, show that the successive orientations of an ant to two different types of stimuli are correlated in a fairly complex way. The object of this section is to propose an hypothesis to explain the fact of correlation, and to consider the dance of the honey-bee in relation to this hypothesis. A discussion of the complexities of correlation will be postponed to a subsequent paper.

The orientation of any insect is, of course, the result of integration between sensory and motor activities. It is possible, theoretically, to distinguish three functional levels of organization within the central nervous system:

1. Sensory mechanisms used in analysing the sensory fields.

2. Locomotory mechanisms used in co-ordinating muscular activity into efficient stepping and turning movements.

3. Taxis mechanisms which intervene between the sensory and motor centres, and by so doing correlate changes in the sensory fields with movements of the animal.

There is some practical justification for these theoretical distinctions: an ant may of course use its sense organs for many purposes other than orientation, which suggests that the sensory centres can function independently of any taxis mechanism: moreover an ant (personal observation) can perform perfectly co-ordinated walking movements even when deprived of its supraoesophageal ganglia, which suggests that motor co-ordination is achieved at a low level of the central nervous system.

In the experiments described, an ant was never subjected to more than one stimulus at a time; at any moment it was performing a compass reaction to either light, gravity or polarized light. All three compass reactions have in common the division of their sensory fields into functionally different parts: when the stimulus lies in one part of the field locomotion is straight forward; when in another part of the field turning occurs. This arrangement keeps the ant moving in one direction relative to the source of stimulation, for as long as the division persists. The device responsible for this functional division is the taxis mechanism referred to above. The hypothesis proposed here is that the maintenance of any one orientation is the result of a particular 'setting' of the taxis mechanism, which persists as long as the run continues.

The experiments show, however, that successive orientations to two stimuli of different types are correlated. This must mean that the 'setting' of the taxis mechanism for the first stimulus must somehow influence the 'setting' for the second stimulus. The explanation suggested here is that the taxis mechanism for each of the three types of orientation considered are, in fact, both functionally and anatomically the same. If this is so, then once the mechanism has been 'set' for one stimulus, substitution of the second stimulus will find this 'setting' already in existence, and the second orientation will inevitably be related to the first.

The existence of a common taxis mechanism, or orientation centre, for all three types of orientation might be expected on grounds of economy; for these three compass reactions have such similar features, that triplication of their underlying nervous components would seem unnecessary.

Consideration of the 'setting' of the orientation centre, raises the problem of how it is initiated. In the experiments, an ant gains no advantage from running in any particular direction; no one orientation is preferred to another, and an individual runs in different directions on different occasions. Presumably experience plays no part in determining the actual direction taken, although an ant may previously have learned to run in a straight line. There are at least two alternative explanations, either the 'setting' is made spontaneously and arbitrarily by the ant itself, or the ant starts running at random, but once started, the stimulus itself fixes the orientation, in that the ant continues along the same course on which it accidentally began; thus the 'setting' would be the direct result of the make up of the sensory

field. This last procedure would be an example of behavioural inertia (Wigglesworth, 1939).

It is valuable to consider what part such behavioural inertia might play in the interchange of different stimuli. When the second stimulus is introduced the ant is already running in a particular direction, and might therefore be expected to continue in this direction while using the second stimulus. Such behaviour would lead to a correlation between the successive orientations. It would not, however, satisfy the experimental observations for the following reasons:

1. The ant does not continue in the same absolute direction as before the stimuli were interchanged, but itself turns.

2. The successive orientations are correlated in a very specific way, which is moreover independent of the angle between the stimuli, and of the ant's direction at the moment of interchange.

These objections may be summarized by saying that after the interchange of stimuli the ant turns through a specific angle, which is directly determined by the angle between the stimuli. Thus if behavioural inertia is to play a part it must act specifically on the taxis mechanism, in a way determined by the first stimulus. This is in no way opposed to the hypothesis already suggested.

The events postulated may now be summarized as follows:

1. The ant starts running at random.

2. The constitution of the sensory field determines the initial 'setting' of the orientation centre.

3. The ant continues to orientate to the first stimulus in a way determined by the 'setting'.

4. The ant orientates to the second stimulus in a way similarly determined by the initial 'setting' of the orientation centre.

The discussion so far has been concerned only with the behaviour of ants under the experimental conditions described. The orientation of an ant under natural conditions is of course a much more complex piece of behaviour, and there is some difficulty in applying the above hypothesis. It is not proposed here, however, to discuss the ant's behaviour any further, but to proceed directly to a consideration of the honey-bee. The behaviour of this insect is perhaps more complicated than that of the ant, but, as the problems of their visual orientation in the field are essentially similar, the same arguments may be applied to both.

Consider first the part that a taxis mechanism must play when a bee is flying freely and performing a light compass reaction to the sun. In the visual field are many sources of stimulation, but if, as may happen, the bee is orientating solely by the sun then the immediate effect of these other stimuli upon the course flown must be inhibited. Similar considerations apply to orientation to polarized light, and also to orientation to gravity in the honey dance. The taxis mechanism must therefore have a selective function, in determining which of many possible stimuli is used.

It is known (Wolf, 1926, 1927, 1931) that bees will also orientate by means of a number of landmarks, such as trees, buildings and mountains. The flight to the hive entrance is also guided by quite a complex pattern of surrounding objects.

Orientation by landmarks has in common with orientation by celestial guides the straight track which the bee makes; it seems a general feature of hymenopteran orientation that if one stimulus is removed the insect can continue on its course undisturbed, utilizing some other part of its environment (see MacGregor, 1948). However, although the behaviour of the bee has the same result whether orientating by terrestrial or celestial guides, there is an important distinction between the two types of steering: when a bee performs a light compass reaction by the sun, or guides itself by plane polarized light from the sky, it does so by keeping its visual field constant with respect to these stimuli; with terrestrial landmarks, on the contrary, it maintains its course by continually changing its visual field with respect to those landmarks, a process necessitated by their nearness. This seems so fundamental a difference that one would be justified in postulating different nervous mechanisms underlying the two types of orientation, and in restricting the term 'compass' to those occasions where the sensory field is kept constant.

Further, while there can be little doubt that the bee must learn to orientate by landmarks, it is possible that orientation by the sun and polarized light need not be learned, for Lindauer (1952) has shown that a young bee which has danced in the hive may fly successfully to the feeding-place on its first flight, when it can have had no previous experience in so orientating. Moreover, as von Frisch has shown, bees which have foraged under overcast conditions do not dance in the hive; a fact which might be interpreted as showing that although bees learn to associate a particular direction with a particular succession of landmarks, they are unable to perform the reverse process, and associate a route flown *entirely* by landmarks with a particular direction, either in flight or in the dance.

It may be suggested therefore that for a bee there are three primary stimuli for orientation—direct sunlight, polarized light from the sky, and gravity—and that the response to landmarks is of a different nature, involving different mechanisms, although producing the same result. If there is a common taxis mechanism for orientation to these three stimuli, then this mechanism must be temporarily unused when the bee orientates to other stimuli, as when flying under cloudy conditions, or when making its way on to the comb prior to dancing. Further, since bees can fly to a food source in a remembered position, the 'setting' of the taxis mechanism must constantly be determined by experience and memory.

The hypothesis may now be summarized as follows: for compass orientations to light, gravity and polarized light there is a single taxis mechanism, the 'setting' of which determines the angle of orientation to any of these three stimuli, and may be produced as a result of experience; the mechanism is selective in utilizing only one component of a complex stimulus situation, and may be left unused when the bee orientates to other stimuli.

If this hypothesis is accepted it may be used to explain the orientation of the honey dance. If the taxis mechanism has been 'set' during the foraging flight, and this 'setting' persists while the returned forager dances on the comb, then the orientation of the straight part of the dance will inevitably be related to the orientation of the flight, whichever of the three stimuli may be used. The dance is

of course not a simple straight line, but a complex pattern; however, only the central part has a constant angle of orientation, and the nature of the dance is such that the bee is continually presented with a stimulus acting in one direction. If, as previously suggested for ants, the action of the stimulus can itself produce a corresponding 'setting' of the taxis mechanism, then a prospective forager would receive precisely the same 'setting' as the returned forager, whose dance it has copied, and this would enable the new forager to follow the same course outside the hive: once on this course the bee may utilize any previous experience of the territory to guide itself along the same course by using landmarks, or make detours involving wide deviations from the true direction of the food source, two procedures in which the common taxis mechanism is temporarily unused.

While the behaviour of the honey-bee has been oversimplified in the above discussion, sufficient has been said to show that there may well be a common mechanism for orientating to the sun, gravity and polarized light, and that the dance is, in part, a device for 'setting' this mechanism in new foragers. The language of the bee is perhaps less perplexing when seen against this background than in the isolation of field observation and experiment.

SUMMARY

1. Von Frisch has shown that in the honey-bee orientation established in relation to one directional stimulus (light) can be transferred to another directional stimulus (gravity or polarized light). In the present work the orientation of ants has been studied in experiments in which one type of directional stimulus has been replaced by another. Light, gravity and polarized light have been used as stimuli.

2. When light and gravity are interchanged, the ant's successive orientations to the two stimuli are correlated. The angle between the track and the stimulus is the same for both orientations.

3. When light and polarized light are interchanged, the ant's successive orientations are again correlated. The actual relationship depends on the experimental conditions.

4. When polarized light and gravity are interchanged there is no significant correlation between successive orientations.

5. It is suggested that in bees and ants there is a single taxis mechanism for orientation to light, polarized light and gravity, and that the 'setting' of this mechanism during an orientation to one stimulus persists and partially determines the subsequent orientation to another stimulus.

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THE ORIENTATION OF ANTS

II. ORIENTATION TO LIGHT, GRAVITY AND POLARIZED LIGHT

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INTRODUCTION

In a previous paper experiments with the ant *Myrmica ruginodis* were described (Vowles, 1954): in these experiments light and gravity, and light and polarized light, were interchanged as orientatory stimuli. Under such conditions successive orientations were correlated in a rather complex way. In the light/gravity substitutions the orientation angle to the second stimulus ($\theta_2°$) was related to the orientation angle for the first stimulus ($\theta_1°$) in one of four ways expressed by the equations:

$$\theta_1 = \theta_2,$$
$$\theta_1 = 360 - \theta_2,$$
$$\theta_1 = 180 - \theta_2,$$
$$\theta_1 = 180 + \theta_2.$$

The causes underlying the occurrence of these four relationships will be examined in this paper.

THE ORIENTATION OF ANTS TO GRAVITY

It has been suggested by Crozier and others that, for many animals, orientation to gravity on an inclined plane is simply the result of the animal turning upwards until it is in stable equilibrium; this being attained when the centre of gravity is within the triangle of support. Work with the beetle *Tetraopes* (Crozier & Stier, 1929) supported this idea, and suggested that the weight of the abdomen was the force most important in disturbing equilibrium. Barnes (1929, 1930*a*, *b*) understood his own results with ants to confirm Crozier and Stier's hypothesis, but recorded also two observations of which he did not recognize the significance:

I. Ants which had their antennae amputated could not maintain any particular orientation when walking on an inclined plane.

II. When walking up an inclined plane ants often oscillated successively left and right of the vertical plane, thus making their track a zigzag placed symmetrically either side of the actual upward direction.

The significance of these observations will become clear later. Similar observations were made with *Myrmica laevinodis* during the present study.

In Crozier's sense orientation to gravity is a simple geotaxis, in which the animal does not need to perceive the direction in which gravity acts, but merely to turn until the strain of maintaining equilibrium is at a minimum. The maintenance by

an ant or a bee of a constant orientation (other than 0° or 180°) on a vertical surface is not, however, of this nature. An ant orientating in this way is usually in unstable equilibrium, for the tip of the abdomen is not trailed on the ground while the insect is walking, which Barnes suggests, and the centre of gravity is always posterior to the support given by the legs. An ant, orientating to gravity, is performing an activity similar to the 'light compass reaction'. It is proposed to call this activity a 'force compass reaction', which may be defined as 'the orientated locomotion of an animal during which its anterio-posterior axis makes any constant angle with the line of action of a physical force; the maintenance of which orientation does not depend on equal bilateral stimulation, or on keeping a stable equilibrium'. Such an orientation is a 'menotaxis' in Kuhn's (1919) terminology.

This type of orientation raises the problem of the sensory mechanisms by which an ant can perceive the direction in which gravity acts. If an ant were sensitive to the magnitude of the rotational forces, due to gravity, which act upon it, this would provide a basis for geo-perception, for these forces vary with the sine of the angle of deviation from the vertical. If the ant perceived only the magnitude and not the direction of these rotational forces, then a confusion might arise between tracks symmetrically placed on either side of the vertical, when the sines of the angles of orientation are equal. These angles are $\theta°$, $360-\theta°$, $180-\theta°$, and $180+\theta°$. It is a confusion between precisely these angles that might be expected from the results of the light/gravity substitutions.

It was therefore decided to investigate the orientation of ants to gravity, and to test particularly the hypothesis that confusion between various orientations does occur.

Apparatus and procedure

The apparatus consisted of a simple, circular turntable, its surface in the vertical plane, which could be rotated about its centre in this plane. The turntable was $8\frac{1}{2}$ in. in diameter, and was placed against a circular scale, which indicated the angle through which it had been turned. The surface of the table was covered with white Bristol board, ruled lightly into 1 in. squares; it gave a good foothold to the ants.

It was found that ants showed little tendency to remain still when on a vertical surface. They would walk in a straight line after very slight stimulation—little was required of the experimenter beyond turning an ant back from the table's edge. A Perspex screen was therefore placed 3 in. away from the table, and parallel to it: the ants' tracks were plotted on this in various shades of 'lipstick'. The red colour of this was convenient, as it disappeared in red light and did not confuse the observer.

On one corner of the Perspex sheet was placed a card on which had been drawn a circle with diameters marked in different colours every ten degrees. By glancing from the ant to this device the observer could make a rough estimate of the actual orientation of the ant, and obtain some idea of the angle through which the table must be turned.

The apparatus was set up in a dark-room in a dim red light. An ant was placed on the turntable and allowed to wander for 2 min. in order to become accustomed

to the conditions. The ant was then gently stimulated and set off in a straight line. Its track was plotted. When it had travelled about 4 cm. the table was smoothly turned into a new position and the track of the ant again plotted. When possible the turning was done when the ant was near the centre of the table. After one such 'run' had been completed the plotted track was given a reference number, and the angle through which the table had been turned recorded. The process was then repeated using a different shade of lipstick for the new track. About four recordings were made with each ant, which was selected at random from three colonies. The angles of orientation for each part of the track were then recorded, being measured clockwise from the vertically downward direction.

Three series of experiments were done in order to test the possible equivalence of various orientations.

I. When an ant was orientating at $\theta°$ it was turned to face in a direction at $180-\theta°$.

II. When an ant was orientating at $\theta°$ it was turned to face in a direction at $180+\theta°$.

III. When an ant was orientating at $\theta°$ it was turned to face in a direction at $360-\theta°$.

The turning was always done the shortest way. With practice the experimenter became fairly accurate in turning the table through the desired angle. Of all the attempts so made about half were accurate to within 15°.

The species of ant used in this and subsequent experiments was *M. laevinodis*.

Results

It was found that an ant would walk in almost any direction relative to the vertical. There was, however, a tendency to avoid walking at 90° or 270° or angles approximating to these, in which position the strain on the ant's legs on one side is presumably maximal.

The orientation angles to gravity before and after turning, were compared for each of the three series. It was found that Series I and II did not differ significantly from each other. In the lower graph of Fig. 1 the orientation angles before and after turning are plotted against each other for the experiments of Series I and II. In the upper graph are plotted the same angles for the experiments of Series III, in which the ant was turned from $\theta°$ to $360-\theta°$, i.e. turned through $2\theta°$. The lower graph will in future be called the control graph, and used for comparison with the upper graph.

Consideration of the control graph shows three effects:

I. A main regression along the line $\theta_1=\theta_2$.

II. A few points lying about the line $360-\theta_1=\theta_2$.

III. A broad band of points lying about the value 180° on the ordinate. When ants were disturbed they often turned and walked in a general upward direction: it is this behaviour which gives rise to this horizontal, broad band of points.

As all these relationships occur on the same graph it was thought that any

statistical estimate of the correlation would have little real significance. The graph may be interpreted qualitatively as showing that:

I. In most cases an ant returns to its original orientation after the table has been rotated.

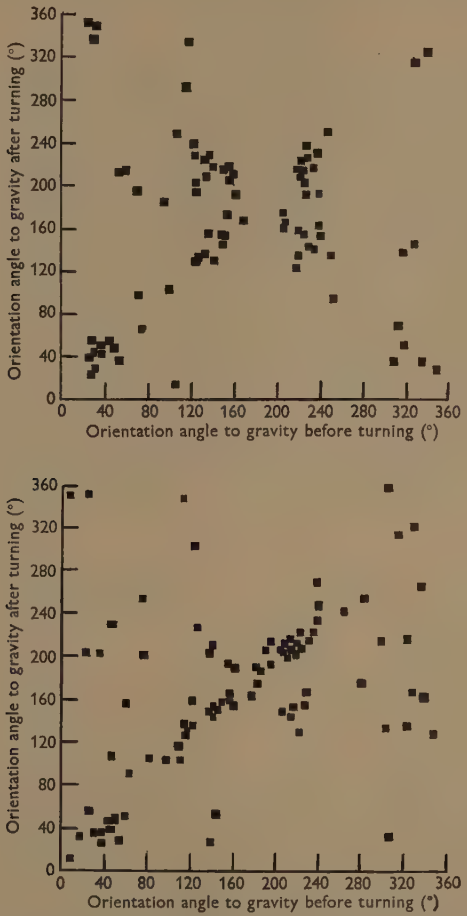

Fig. 1. The orientation to gravity of *M. laevinodis*. Upper graph: when table is turned through twice the angle between the track and the vertical. Lower graph: control series.

II. Occasionally an ant turns to a new orientation, expressed by $360-\theta_1=\theta_2$, after rotation of the table.

III. There is a tendency for ants to walk upwards after being disturbed by movement of the table.

The main importance of this control graph lies in providing a comparison with the upper graph. Consideration of the upper graph shows the points to be scattered about the two arms of a cross, the equations of which could be expressed by:

$$\theta_1 = \theta_2,$$

and

$$360 - \theta_1 = \theta_2.$$

The first equation would be the result of the ants returning to their original orientation after the turning of the table; and the second equation the result of the ants turning very little after the table had been rotated.

There are no points at the centre of the graph because when an ant was orientating at about 180° the table did not have to be turned, and the ant was not therefore disturbed. It was thought that such results, where the ant walks straight on, would not contribute to the analysis, and they were omitted. In addition to the points lying along the arms of the cross there are others scattered apparently at random, and others showing that an ant turned and walked upwards after the rotation of the table.

No statistical analysis of the graph has been attempted, as it is considered that the relationships are fairly obvious.

It was expected that the points falling about the line $\theta_1 = \theta_2$ would come from those results when the table had not been turned through the required angle $(2 \times \theta_1)$, while those on the line $360 - \theta_1 = \theta_2$ would come after successfully turning the table. This was found not to be so, as is shown in Table 1 below.

Table 1

Error in turning table	0–15°	15–30°	30–45°	45–60°	60–75°	75–90°	Total
No. of points on the line $\theta_1 = 360 - \theta_2$	17	10	3	3	2	1	36
No. of points on the line $\theta_1 = \theta_2$	16	10	4	1	2	2	35
No. of points on neither	5	0	4	0	0	1	10

The first orientations appear not to influence which of the two possible new orientations shall be chosen, as can be seen from the graph itself.

An attempt was made to calculate the accuracy of the gravity orientation from the control experiments. A graph was plotted of angle through which the ant turned/angle table turned. Again a main regression was found corresponding to the line $\theta_1 = \theta_2$ on the original control graph: but the other points were now scattered widely over the whole area of the graph. Those points obviously not falling along the main regression line were then omitted from the calculations. The calculated regression was

$$y = 17{\cdot}69 - 0{\cdot}8545x.$$

The standard error of 17·69 was $\pm 9{\cdot}29$, and this constant therefore does not differ significantly from 0.

The standard error of 0·8545 was ± 0·0649, and this figure is therefore significantly less than 1. This implies that the ants turn through slightly less than the angle through which the table was turned.

90% of the points fell within ± 33° of the regression line. This may be taken as an indication of the possible accuracy of orientation to gravity.

Discussion

The experiments show that if an ant is orientating to gravity, and is turned away from its original heading, it will usually return and again take up its original orientation. However, in the experiments, when an ant had been orientating at $\theta°$ and was turned toward or past a position in which it would have been heading at $360-\theta°$, it only resumed its original orientation on about half these occasions; on the other half it took up a new orientation at $360-\theta°$. There was also a tendency for ants to do this in control experiments. This agrees with Barnes's observations that ants often oscillate, giving a zigzag path about the vertical. This was also seen in *M. laevinodis*.

It seems therefore that ants somehow confuse orientations lying symmetrically on either side of the vertical. This could be due to either central or sensory factors or both. It is possible, however, to imagine a situation in which such confusion would be the direct result of the physical factors involved in georeception. Suppose, for example, that the ant was sensitive to the distorting force due to gravity upon some part of its body. These forces will vary with the orientation of that part of the body relative to the vertical: the rotational force will be maximal when the axis is at 90° to the vertical, and minimal when it is parallel. The size of the rotational force is proportional to the sine of the angle between the axis and the vertical. Therefore for any one position in each quadrant there is a corresponding position in each other quadrant where the rotational forces are the same. If the ant could distinguish the magnitude but not the direction of these rotational forces it would confuse four orientations placed symmetrically about the vertical. However, the distorting force due to gravity also has a longitudinal component acting along the axis of the part of the body involved: the direction of this component is directly opposite when the axis points in a general upwards, or a general downwards direction. Therefore if the ant was sensitive to the magnitude but not the direction of the rotational force, and to the direction but not the magnitude of the longitudinal force it would confuse two orientations placed symmetrically left and right of the vertical.

If this hypothesis is taken as a working basis for experiment one must consider various joints of the body across which strain could be measured. Those considered here are

(1) The abdomen to the thorax.
(2) The thorax to the head.
(3) The limbs to the thorax.
(4) The scape of the antenna upon the head.
(5) The funiculus of the antenna upon the scape.

The experiments described below were designed to locate the georeceptor within the ant.

LOCALIZATION OF GORECEPTOR

Apparatus and procedure

Two standard methods used in such experiments—extirpation of parts, and splinting of joints—could not be used here. All the parts of the ant to be considered have important functions other than geosensory, and both extirpation and splinting caused general behavioural disturbance, which rendered the operated individual unsuitable for experiment. Moreover, it is often unwise to make inferences about the normal function of an organ based solely on observations of an animal's behaviour after it has been deprived of that organ.

The method used here was to vary the actual rotational forces upon different parts of the ant. This was done by cementing a small particle of soft iron to the part of the ant to be studied, and while the insect was walking on a vertical surface subjecting it to a magnetic field. Care was taken to use a minimum of shellac cement, particularly on the antennae.

The apparatus consisted of a flat board, 25 cm. wide, fixed in a vertical position. The board was painted dull black, and on its surface a filter paper, lightly pencilled into 2 in. squares, was glued, to provide a good foothold for the ants and a good background for the observer: being circular and of large diameter (23 cm.) the filter paper had no sharp corners which might have acted as landmarks for the ant. At either end of the transverse axis of the board was placed a large flat-wound solenoid, mounted on brass formers, 8 in. in diameter and $\frac{1}{2}$ in. wide. The solenoids were placed with their planes at 90° to the surface of the board.

Each solenoid had 100 turns of wire, and a resistance of 4 Ω. They were connected in parallel with a 4 V. accumulator. The current passing through each of them was 1 A. This arrangement gives a fairly uniform magnetic field of strength approximately 3 G. The lines of force lay horizontally across the surface of the board. The effect of the magnetic field was to produce a rotational couple tending to turn an iron rod into the direction of the field. This couple acted on the same direction, clockwise or anticlockwise, when the axis of the iron rod lay in diagonally opposite quadrants; and in the opposite direction in adjacent quadrants. The apparatus was set up in a dark-room, in a dim red light, with the earth's magnetic field acting in a plane at 90° to the surface of the board: the earth's field therefore could not influence orientation in the plane of the board.

An ant prepared for the experiment was placed on the filter paper, and was allowed to wander at random for 2 min. The escape reaction was then released, as in the turntable experiments. When an ant had travelled for a few centimetres the magnetic field was switched on and the behaviour of the ant observed. The procedure was then repeated with the field on initially, and switched off while the ant was running. About six such observations were made with each ant.

Results

It was observed that ants with the iron filings cemented to them did not behave differently from normal ants when on the vertical surface, when the magnetic field was off or when it was on all the time. It seems that ants can orientate satisfactorily under both conditions. The results of the experiments are summarized in Table 2.

The fact that some sort of response was shown by the ant in all cases, except when filings were on the thorax, suggests that the magnetic force exerted was sufficiently strong to be perceived by the ant, even when it had no effect on orientation. An attempt was made to calculate this force and to compare it with the forces due to gravity: a number of assumptions had to be made about the dimensions of the filings used, which were not uniform, the inductance of the iron, which was not

Table 2

Experimental conditions	No. of ants	No. of observations	Effect on the ant of switching the magnetic field on or off
Iron filing on abdomen	8	53	No effect on orientation. An ant sometimes swerved slightly, and at other times stopped and made attacking movements
Iron filing on head	12	64	As above, but ants were more sensitive to the change of field
Iron filing on thorax	3	18	No effect observed
An iron filing on the funiculi of both antennae	12	74	On about half the occasions on which the field was changed the ant stopped, and often cleaned its antennae. On the remaining occasions it smoothly changed direction and continued in a straight path
An iron filing on the scape of both antennae	12	69	Sometimes stopped temporarily, as described above, and at other times made a large swerve and then returned to its original orientation

known, the size of the ants, which varied, and the exact position in which the filing was attached to the ant; such assumptions of course decrease the accuracy of the estimation, but it may be said that the maximum magnetic couple is of the order of 10× the rotational force due to gravity upon one antenna. It seemed possible therefore that this force was too small, compared with the weight of the head and abdomen, to influence orientations depending on these members. The fact that the magnetic force caused other behavioural effects, such as swerving, biting and stopping, argues against this possibility, but does not eliminate it. A few experiments were therefore done using a strong bar magnet: an ant with filings on either its head or abdomen was allowed to run on a vertical surface, and then the magnet was suddenly placed diagonally behind it; the force exerted was strong enough to pull the head or abdomen sideways; this usually caused the ant to stop, but occasionally it would continue to run, and in such cases preserved its old orientation: this supports the hypothesis that the head and the abdomen are not concerned with orientation to gravity in ants.

The results can be interpreted as follows: the georeceptor is situated somewhere between the funiculus and the scape; the perception of the direction in which gravity acts, relative to the ant, depends on the rotational force exerted on the funiculus; when an iron filing is present on the funiculus, and a magnetic field is operating on the filing, the stimulus for orientation is the sum of the rotational forces due to gravity and the magnetic couple. Thus, when a magnetic field is switched on or off an ant turns until in a position where the rotational forces are restored to their original value. When an iron filing is present on the scape, change of magnetic field may cause actual movement of the antenna, leading to a temporary change in the rotational forces on the funiculus, which persists until the ant restores its antenna to its normal position; such a change would lead to temporary disorientation and swerving as seen in the experiments.

When an ant is running freely on a vertical surface it holds its antennae in a fixed position. The scape is held diagonally forward and outward, at about 30° to the anterio-posterior axis of the ant; the funiculus is held more nearly parallel to this axis, but is turned slightly outward from it, particularly at the club-shaped tip. The relative positions of the antennae to the head appear the same at all orientations: the rotational forces upon the funiculus are therefore not equal for two orientations lying symmetrically either side of the vertical, for the two symmetrical positions for the ant are not symmetrical for the funiculus. The confusion between such orientations cannot, therefore, be due to equal rotational forces upon the funiculus, and needs another explanation.

It seemed possible that the explanation might lie in the interaction of the two antennae. In the experiments so far described an iron filing was placed on both antennae. It was not known, however, what would happen if an iron filing was placed on only one of the antennae, when the two antennae would be unequally influenced by a change in the magnetic field. Some experiments were therefore done with ants which had an iron filing fastened to only one antenna. The experimental procedure was then the same as in the previous experiments.

It was found that when the magnetic field was switched on or off an ant was usually disturbed in some way, sometimes it smoothly changed direction, and sometimes it swerved but then returned to its original orientation. The results are summarized in Table 3. If, when the ant smoothly changed direction, the two parts of the track were symmetrically placed either side of the vertical it was assumed that the ant had turned spontaneously making part of a normal zigzag path, and in the Table such behaviour is counted as if the ant had not turned.

If the magnetic field was changed more than once during a single run it was seen that an ant sometimes responded to only one of these changes by turning; whether the operative change was the first or the second could not be predicted, it varied both with different ants and with the same ant on different occasions. No significant difference was observed between turns caused by the presence of the iron filing on one antenna, and those when both antennae were affected.

These results suggest that the georeceptors function independently in the two antennae, and are used singly, the receptor on one side being dominant at any one

time; if this is so it is a very unusual condition. A change from one antenna to the other may possibly occur during an orientated run, causing a change from the original to its equivalent direction. When an ant is orientating at $\theta°$ the magnitude of the rotational force due to gravity on one funiculus is equal to similar forces on the other funiculus when the ant is orientating at $360-\theta°$. If one assumes that equal forces on the two antennae produce the same effect on the taxis mechanism, then the equivalence of two such orientations could be due to the use of one antenna for one orientation and the other for its equivalent; the zigzag path would then be the result of successive changes from one to the other organ. The turntable experiments suggest that the change can be initiated by swinging the ant toward or past the vertical.

Table 3

Antennae bearing iron filing	Ant	No. of occasions when ant turned	No. of occasions when ant did not turn	Total
Right	A	11	5	16
Right	B	6	10	16
Right	C	6	4	10
Left	D	4	6	10
Left	E	6	5	11
Left	F	7	1	8
Total	6	40	31	71

THE STRUCTURE OF THE GEORECEPTOR IN ANTS

The experiments using iron filings located the georeceptor between the scape and the funiculus of the antennae. The only organ present in this region, which could fulfil the functions of a georeceptor, is Johnston's organ, which is situated in the pedicellus. This organ, which consists of scolopidia or modified chordotonal organs, was first described by Johnston (1855) in *Culex*. Child (1894*a*, *b*) showed it to be present in Hymenoptera in a poorly developed condition. This was confirmed by Eggers (1923) and Snodgrass (1924).

McIndoo (1922) working on Johnston's organ in the honey-bee showed that in these insects it took a peculiar form. He describes the scolopidia (about eighty in number in the worker) as attached at their distal ends to a series of chitinous knobs developed on the outer surface of the intersegmental membrane. These knobs are arranged in a ring around the outside of the base of the second segment of the funiculus (the pedicellus being the first). The intersegmental membrane is much strengthened by strands of flexible chitin, between which the scolopidia pass.

An investigation was made of the form that Johnston's organ takes in the ant *Formica rufa*, the antennae of which are more amenable to sectioning than those of smaller species. The structure closely resembles that of the bee. The scolopidia, about twenty in number, are arranged in a ring around the pedicellus. Their proximal ends are attached to the wall of this segment. The distal end of each scolopidium is attached to a chitinous knob on the outer surface of the interseg-

mental membrane. These chitinous knobs are posterior projections from a narrower chitinous ring, which passes completely around the outside of the base of the second segment of the funiculus. The inner margin of the ring is set, tightly, in a corresponding groove in the wall of the second segment. The outer margin lies in the intersegmental membrane.

The intersegmental membrane is thick, and strengthened with bands of chitin. It is not very convoluted, however, when compared with that of other segments: this restricts the range of movement of the second segment upon the pedicellus, a fact which can be verified by observation of the relative mobility of the various segments.

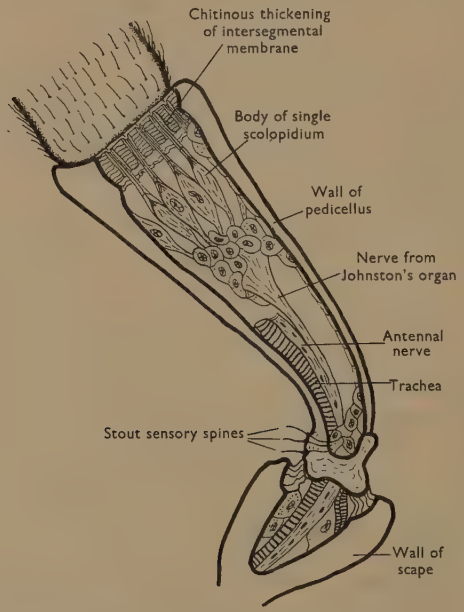

Fig. 2. Johnston's organ in *Formica rufa*. The dorsal wall of the pedicellus is removed to show the scolopidia.

The thick intersegmental membrane may have a twofold function. First, it prevents too wide a range of movement of the funiculus on the pedicellus, and secondly, it may take some of the strain across the joint between the two segments. Both of these activities would protect the scolopidia. It is generally accepted that the normal function of chordotonal organs, or scolopidia, is to respond to changes in tension. In Johnston's organ changes in tension could be produced by slight changes in position of the funiculus relative to the pedicellus. Different orientations to gravity will result in different rotational forces on the funiculus. These will tend to displace the funiculus by different amounts; the actual size of the displace-

ment is restricted by the intersegmental membrane, thus keeping it within the range of sensitivity of the scolopidia, and also preventing large displacements which might damage them.

THE ORIENTATION OF ANTS TO LIGHT

The investigation of the ant's force compass reaction to gravity showed that pairs of orientations at $\theta°$ and $360-\theta°$ were equivalent to each other. This alone would not explain the occurrence of the four relationships obtained in the light/gravity substitution experiments. It was therefore decided to make a study of the ant's reactions to light, with the object of ascertaining if equivalent orientations existed in the light compass reaction also. As the outward and return tracks of ants going to and from their feeding place are orientated at 180° to each other, and since these tracks are both orientated relative to light from the sun (on occasion), it was thought that perhaps for the light compass reaction orientations 180° apart might be equivalent to each other.

Apparatus and procedure

The experiments described here were very similar to those for the equivalent investigation of gravity orientation. The turntable described for those experiments was placed so that its surface was horizontal and could be rotated in a horizontal plane. The apparatus was set up in a dark-room, with an electric bulb (210 V. 60 W.) placed the far side of the room (about 3 m. away) on a level with the turntable. This bulb was suitably screened so that its beam of light was restricted to the turntable, and did not reflect from other objects in the room. The light could be switched on and off by a foot-operated switch. A dim, red safe-lamp was placed by the turntable so that the ants could be seen while the light was off.

A Perspex screen, on which to plot the tracks, was found unsatisfactory for these experiments, and the track of the ant was therefore plotted directly on the surface on which the ant walked. The surface of the table was covered by a sheet of cartridge paper, lightly pencilled into 1 in. squares. The track of an ant was plotted by marking lightly, in pencil, the points at which it entered and left a square: this being done when the ant was one square ahead. A fresh sheet of paper was used for each ant. In addition to the squares on the paper a diameter was lightly pencilled in every 30°, to assist in estimating the angle of orientation. The paper was attached to the turntable by four low mounds of Plasticine, which cast little shadow.

An ant was placed on the turntable and allowed to wander at random for 2 min. to become accustomed to the conditions. The escape reaction was then elicited by a gentle touch from a glass rod. The ant ran in a straight line, and its track was plotted. While it was running the angle of orientation $\theta°$ was estimated and the angle through which the turntable had to be turned was calculated. After the ant had travelled about 4 cm. the light was switched off, the table turned rapidly but smoothly through the calculated angle, and the light switched on again. The ant usually turned during and after the turning of the table, and then continued in a straight line. The track was again plotted. The track was then drawn in in detail, and

the angle through which the table had been turned recorded. The whole procedure was then repeated. On an average five tracks were recorded for each ant.

The angles of orientation for each track (measured clockwise from the direction of the light) were then recorded before and after turning.

Three series of experiments were done:

(*a*) Attempting to turn the table through $180-2\theta°$. This would turn an ant facing at $\theta°$ to head in a direction at $180-\theta°$.

(*b*) Attempting to turn the table through $2\theta°$. This would turn an ant facing at $\theta°$ to head in a direction at $360-\theta°$.

(*c*) Turning the table through 180°. This was done alternately clockwise and anticlockwise.

N.B. $\theta°$ is the acute angle between track and light direction, and is always less than 90°. Both series (*a*) and (*b*) involved turning in both directions.

The details of the number of readings taken are given in Table 4.

Table 4

Series	No. of ants used	No. of readings
1	13	52
2	8	35
3	8	30
Total	29	117

Results

Graphs were plotted, for each series of experiments, of angle track to light before turning/angle track to light after turning. No significant difference was observed between the results of series (*b*) and (*c*). These two series were therefore combined, and will in future be referred to as controls.

The graph for these control experiments is shown in the lower part of Fig. 3.

The control graph shows points grouped about the main regression line $\theta_1=\theta_2$, with some few other points scattered outside the main band. Some of these scattered points seem to lie about the line $\theta_1=360-\theta_2$, although the relationship, if it exists, is not a close one. If all the points obviously not in the main band are considered their scatter seems to be random. When the results corresponding to these points are examined it is found that in all but three cases the table was turned through a large angle, more than 160°. Turning the ants through large angles seems to disturb them rather more than small angles, and there is then a tendency for them to run rapidly straight on, rather than taking up a particular orientation. It is thought that this tendency is the reason why some of the scattered points lie along the line $\theta_1=360-\theta_2$, and that the arrangement is not due to any equivalence of orientations.

The conclusion drawn from the control experiments is that if the turntable is turned so that the ant is swung away from its original heading, it usually returns to its original orientation. As at least thirteen points out of sixty-five fall well outside the main band of points on the graph, the accuracy of the relationship was calculated

by finding the area on either side of the line within which 80% of the points fell. No statistical calculation was made. The error in turning, made by the ant, calculated on this basis was $\pm 15°$. This indicates the possible inaccuracy of a light compass reaction in this species (*M. laevinodis*).

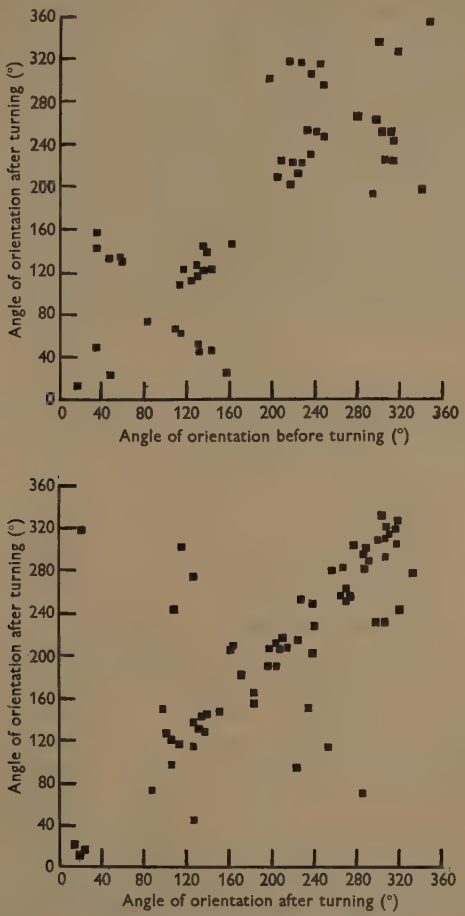

Fig. 3. The orientation to light of *M. laevinodis*. Upper graph: when table is turned through 180° minus the angle between the track and the direction of light. Lower graph: control series.

The upper part of Fig. 3 shows the relationships for series (*a*) where the turntable was turned through $180-2\theta°$. The main regression of $\theta_1 = \theta_2$ is still found here, but in addition some points lie about the two lines represented by $\theta_1 = 180 - \theta_2$. This last relationship would be the result of ants not turning after the table had been accurately rotated. In fact it was found that when the table had not been

turned through the required angle the ants themselves often turned to take up a new orientation. This is shown in Table 5. Points are taken to show the relationship when they fall within 15° of the regression line; when they fall near both lines they are allocated to the nearest.

These results show that if an ant is orientating at $\theta°$ to the light, and is turned toward or past a direction in which it would be heading at $180-\theta°$, it then turns either to renew its original orientation, or to take up a new orientation at $180-\theta°$. This indicates that the two orientations are equivalent in the light compass reaction, under the conditions used.

This equivalence is extremely surprising, for nothing in the normal behaviour of the ant would indicate such a result. Moreover, in the two equivalent orientations the light shines into the same compound eye, and nothing yet known about the structure of the eye or the central nervous system provides a basis to explain the phenomenon. It was therefore decided to investigate the structure of the ant's compound eye, in case any structural linkage of ommatidia occurred, and also to check upon the accuracy of normal light-compass orientations.

Table 5

Error in turning table	0–15°	15–30°	30–45°	45–60°	60–75°	75–90°	90–105°	Total
No. of points on the line $\theta_1=\theta_2$	6	7	3	5	1	0	1	23
No. of points on the line $\theta_1=180-\theta_2$	4	7	9	2	1	0	0	23
No. of points on neither line	2	1	0	2	1	0	0	6
Total	12	15	12	9	3	0	1	52

THE STRUCTURE OF THE COMPOUND EYE OF *M. RUGINODIS*

Studies by Santschi (1923) and Werringloer (1932) have shown that the eyes of ants have a typical eucone structure. The rhabdome is very fine, and the number of retinal cells around each rhabdome is reduced to seven. Sections cut through the eye of *M. ruginodis* confirm this description.

The individual eye contains between two and three hundred ommatidia. The field of view of each eye is from directly forwards to within 10° of the body's longitudinal axis behind. This means that there is a blind arc of 20° behind the ant. As an ant can run quite satisfactorily directly away from a lamp, it can presumably orientate by keeping light out of any ommatidium. The field of view in the vertical plane is from vertically upwards to about 43° below the horizontal.

The angle between adjacent ommatidia in the same plane varies from 6° to 12° with a mean value (from sixty-eight measurements) of 9°. The accuracy of the orientation to light was previously shown to be $\pm 15°$. In the experiments described the angle subtended by the light-source was 2°.

In experiments on the light-compass reaction with other insects (von Buddenbrock, 1931, 1935; von Buddenbrock & Schulz, 1933) a point source of light was always used. It was shown that corrective turning movements occur when the light moves

from the originally illuminated ommatidium into an adjacent one. The conditions, however, do not allow us to distinguish between the two possibilities:

(*a*) That turning movements occur when light first shines into adjacent ommatidia;

or (*b*) That turning movements occur only when light has wholly left the original ommatidium.

In the experiments described here, the first possibility would lead to an accuracy of orientation of ± the largest ommatidial angle minus the angle subtended by the light source, this figure being 10°. The second possibility would lead to an accuracy of orientation of ± the largest ommatidial angle plus the angle subtended by the light-source, this figure being 14°. This suggests that the second possibility is the more correct. More experiments on this subject are, however, needed.

The structure of the eye does not reveal any basis for the equivalence of two orientations. Neither does this equivalence have any manifestation in the ant's normal behaviour; it is apparently brought forth only under the experimental conditions used. No explanation can as yet be given for it.

THE ORIENTATION OF ANTS TO POLARIZED LIGHT

The sensitivity of *Myrmica ruginodis* to polarized light has previously been demonstrated (Vowles, 1950). It is now known that *Lasius niger* (L.) also possesses this sensitivity (Carthy, 1951). A histological examination of the eye of *Myrmica ruginodis* was made in order, if possible, to identify the insect's analyser. The study revealed that no analyser was present either in the crystalline cone, or in the corneal lens. The analysing structure must therefore be in the rhabdome, the dimensions of which were too small to allow any satisfactory observations to be made.

The work of Menzer & Stockhammer (1951) shows that each rhabdomere acts as an analyser, and that in the eye of the bee the planes of analysis of the rhabdomeres are arranged tangentially about the main axis of the rhabdome in each ommatidium. This confirms the hypothesis of Autrum & Stumpf (1950), which was based on electrophysiological studies. There is no reason to suppose that the mechanisms of the ant's eye differ from the bee's. There are, however, only seven rhabdomeres to each ommatidium in the ant, and presumably the analysers are arranged in a heptagon around the axis of the rhabdome, rather than in an octagon as in the bee.

The experiments with *M. ruginodis* used only vertical beams of plane-polarized light. Such beams could stimulate only those ommatidia looking vertically upwards. In the ant, where the ommatidial angle is so large, only one ommatidium from each eye fulfils this condition. It appears therefore that ants can orientate to the plane of polarization of light when only one ommatidium in each eye is stimulated.

The angle between the sides of a heptagon is approximately 51°. Assuming the sides of the figure to be numbered consecutively 1–7, then the angle between side 1 and side 4 or side 5 is 27°. The range over which one retinula cell is more strongly stimulated than any other (when the plane of polarization most nearly coincides with its own plane of analysis) is therefore 27°. In the experiments with polarized

light the accuracy of orientation was found to be $\pm 27°$. While the exact coincidence of the two figures is unexpected, their closeness would lead one to suggest that corrective turning movements occur only after the plane of polarization has been rotated until the originally maximally stimulated retinula cell is no longer the most highly stimulated. This could be due, as von Frisch suggests, to a change in the pattern of stimulation of the ommatidium as a whole; there is, however, no evidence to support this hypothesis. It is equally possible that orientation to polarized light is performed by keeping the stimulation of a single retinula cell maximal; all that such a process requires is that the maximally stimulated cell is maintained so, by inhibiting any turning movements caused by stimulation of other retinal cells, until they are more strongly excited. This hypothesis would need a simpler neurological mechanism than does that of von Frisch, and the mechanism parallels that required for the light compass reaction.

If the above hypothesis is accepted it means that the ommatidium is not a functional unit, except in physico-optical terms. It should be stressed that the experiments so far performed on the light compass reaction do not allow us to decide whether the retinula cells in a single ommatidium are acting in unison, or individually or successively. The possibility remains that even in the light compass reaction it is a single retinula cell which acts as the 'fixation' point in the eye. If this were so, then orientation to light and to polarized light would involve very similar mechanisms.

GENERAL DISCUSSION

The main object of the experiments described in this paper was to investigate orientation to light and to gravity, in the hope that the results obtained would help to clarify the causes of the complexity of the correlation between successive orientations to these two stimuli. The experiments on geo-orientation show that an ant on a vertical surface may confuse orientations related by the equation $\theta_1 = 360 - \theta_2$. This confusion, or equivalence, between two orientations does occur under natural conditions. It was suggested that an ant uses the georeceptor in only one antenna at one time, and that the taxis mechanism cannot distinguish between the information received from the two antennae.

In the light compass reaction of ants, under the experimental conditions used, there is a confusion, or equivalence, between orientations related by the equation $\theta_1 = 180 - \theta_2$. This confusion is not shown under natural conditions, and no explanation can be given for it. It is suggested that, as in orientation to gravity, the taxis mechanism cannot distinguish between information received from the visual centres during two equivalent orientations.

It was suggested in a previous paper that compass reactions to light, gravity and polarized light were controlled by a common taxis mechanism, the 'setting' of which determines the actual direction taken relative to any of these stimuli. The mechanism, presumably, functions in response to information received from the sensory centres. Since the pair of equivalent orientations for gravity differ from the pair for light, the equivalencies cannot be due to defects in the taxis mechanism

alone; for if they were, one would expect the same equivalencies for both stimuli. The defect must be in the information received by the taxis mechanism, and it is suggested that the two equivalencies for each stimulus occur because the information for two equivalent orientations is the same. Further, if the taxis mechanism cannot distinguish between information received from different sensory centres, then successive orientation to two different types of stimuli will be correlated in a complex way. Consider four examples of how this might happen for light and gravity:

(1) An ant on a vertical surface is orientating at $\theta°$ to gravity, using the Johnston's organ in its right antenna. The taxis mechanism is 'set' in the corresponding way.

Light is now interchanged with gravity as the orientatory stimulus. The taxis mechanism maintains its 'setting', and therefore the ant turns until it orientates to the light at $\theta°$. The successive orientations are therefore related by the equation $\theta_1 = \theta_2$.

(2) An ant on a vertical surface is orientating at $360 - \theta°$ to gravity, but is using its left antenna: this is the equivalent orientation to (1) above. The taxis mechanism is therefore 'set' in exactly the same way as in (1) above.

Light is again interchanged with gravity, and as the 'setting' of the taxis mechanism is the same as before, the ant again turns until it orientates at $\theta°$ to the light. The successive orientations are therefore related by the equation $360 - \theta_1 = \theta_2$.

(3) An ant on a vertical surface is orientating at $\theta°$ to gravity, using its right antenna as in (1). The taxis mechanism is 'set' in precisely the same way as in the two examples already given.

Light is now interchanged with gravity, and the taxis mechanism maintains its 'setting'. However, a confusion now arises, so that it cannot distinguish between $\theta°$ and $180 - \theta°$; so that the ant turns until orientating to light at the latter angle. The successive orientations are therefore related by the equation $\theta_1 = 180 - \theta_2$.

(4) An ant on a vertical surface is orientating at $360 - \theta°$ to gravity using its left antenna, as in (2). The taxis mechanism is 'set' precisely as in all the other examples.

Light is now interchanged with gravity as the orientatory stimulus. However, the confusion again arises, as in (3), between equivalent orientations, and the ant again turns to orientate at $180 - \theta°$. The successive orientations are therefore related by the equation $360 - \theta_1 = 180 - \theta_2$, which becomes $\theta_1 = 180 + \theta_2$.

The same relationships will be found when the ant orientates to light first. It will be seen therefore, that if the taxis mechanism does not distinguish between information from different sensory centres, or from the same centre when the ant orientates in equivalent directions, then successive orientations will be correlated in precisely the four ways demanded by the experiments on the interchange of light and gravity.

While the complex correlations following the interchange of light and gravity can be analysed as shown above, the results of interchanging light and polarized light cannot be so analysed. The experiments described here in no way clarify the complexity of the latter results. Consideration of the experiments on the interchange of light and polarized light suggests, however, the direction which further investiga-

tions might take: in the experiments there was in fact a double change of stimulus; for the light not only changed from normal to polarized, but also changed its direction from horizontal to vertical. It is not known what effect a vertical displacement of the light source has on an ordinary light compass reaction, although under natural conditions an ant can make good a general direction across a rough terrain, on which it continually rolls, pitches and yaws. Nor is the effect on orientation known of rotating the plane of polarization of a beam of light which is other than vertical, although bees can learn to orientate to such a stimulus. Until such effects have been analysed it would be unprofitable to speculate about the results from interchanging light and polarized light.

In all the experiments described here, the line of action of the orientatory stimulus lay in a plane at right angles to the dorso-ventral axis of the ant; variations in direction of the stimuli were always in this plane, as were the movements of the ant; thus the experiments were concerned only with the control of yawing. In all the observations made an ant never turned through more than 180°, either after change in direction of a single stimulus, or after interchange of different stimuli. This means that an ant always turned to its second orientation through the smallest angle possible: a similar result was obtained by von Buddenbrock & Schulz (1933). This implies that the sensory field has been divided into two functional halves, stimulation in one half causing turning in one direction, and stimulation in the other half causing turning in the opposite direction; the actual line of division corresponding to the line of action of the original stimulus. The maintenance of this functional division is presumably a property of the taxis mechanism.

Orientation to polarized light, however, differs from orientation to light and gravity in one important respect: for while the last two stimuli are vectorial in character, the plane of polarization is not. Thus, if the plane of polarization is rotated through 180° the stimulus situation remains unchanged, although for light and gravity this is not so. The plane of polarization apparently lies right across the visual field, and cannot be said to act in any particular half of it. Therefore although all three types of orientation depend on keeping the sensory field constant, the method of analysing the field for polarized light must differ from that used in the light compass reaction. More work on this subject is still needed, but there is already good reason to suppose that the same taxis mechanism underlies all three types of orientation.

The work described was done in the Department of Zoology and Comparative Anatomy at Oxford, while the author held a Junior Research Grant from the Department of Scientific and Industrial Research.

SUMMARY

1. The orientation of ants to gravity has been investigated, and it is shown that orientations symmetrically placed on either side of the vertical are confused.

2. The georeceptor is located in the antenna, and is probably Johnston's organ. Only one antenna is in use at any one time during geo-orientation.

3. The light compass reaction has been investigated and it is shown that orientations $\theta°$ and $180-\theta°$ are confused. No explanation can be offered for this.

4. The results are discussed in relation to the hypothesis that ants have a taxis mechanism common to all senses. It is suggested that the function of this mechanism is to set up a temporary labile symmetry of the ant with respect to orientatory stimuli.

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THE INORGANIC COMPOSITION OF THE BLOOD OF *MYTILUS EDULIS* AND *ANODONTA CYGNEA*

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I. INTRODUCTION

The fresh-water Lamellibranch, *Anodonta cygnea*, is remarkable for the low concentration of solutes in its blood which has an osmotic concentration of only one-twentieth of that of sea water. Although a number of analyses of *Anodonta* blood have been published (De Waele, 1932; Hayes & Pelluet, 1947; Florkin & Duchâteau, 1950), no complete analysis has been available. This paper reports the results of analyses of *Anodonta* blood for all the major inorganic constituents, sodium, potassium, calcium, magnesium, chloride, sulphate, phosphate and total carbon dioxide. For comparison analyses have also been made of the blood of a marine Lamellibranch, *Mytilus edulis*. In spite of the great difference in total concentration between the two body fluids the concentrations of calcium and total carbon dioxide in *Anodonta* are similar to those in *Mytilus*, although all the concentrations of the other ions, sodium, potassium, magnesium, chloride and sulphate are reduced in approximate proportion to the total concentration. It is concluded, from a consideration of the solubility products of calcium carbonate in ionic solutions, that the body fluids of both animals are saturated with respect to aragonite.

II. MATERIALS AND METHODS

M. edulis from Plymouth were kept in a sea-water aquarium at 10° C., Cl 20·3‰, pH 7·8.

Calcium, carbon dioxide and pH measurements were made on animals which had been kept in separate tanks of fresh sea water with a plentiful supply of air. These tanks contained about 2 l. of sea water per animal and were kept at 10° C. Under these conditions the pH of the tanks could be kept in the range 7·9–8·0.

Anodonta cygnea were kept in a current of Cambridge tap water: 12–15° C., pH 7·5.

Blood was usually collected by syringe from the heart after the contents of the pericardium had been removed. The larger quantities of blood required for potassium, magnesium and phosphate determinations in *Anodonta* were collected by making an incision from the floor of the pericardium, anterior to the organ of Bojanus, into the foot and allowing the blood to drain into a beaker. Debris and corpuscles were removed by centrifugation. Blood required for pH and carbon dioxide determinations was centrifuged under liquid paraffin.

Sodium was estimated gravimetrically as sodium zinc uranyl acetate. Potassium was precipitated as potassium silver cobaltinitrite and estimated by titration with

ceric sulphate. Calcium was precipitated as calcium oxalate and estimated by titration with ceric sulphate. Magnesium was estimated iodometrically after precipitation as magnesium oxinate. The methods used were those of Robertson & Webb (1939) and Cruess-Callaghan (1935).

Mytilus chloride was estimated by direct titration with silver nitrate, using potassium chromate as indicator, after the protein had been removed by precipitation with zinc sulphate and barium hydroxide.

Chloride in *Anodonta* blood was estimated by Conway's microdiffusion method (Conway, 1947). Sulphate was estimated iodometrically by the method of Webb (1939). Phosphate was estimated by the colorimetric method of Fiske & SubbaRow (1925), carbon dioxide by Conway's microdiffusion technique. pH measurements were made by glass electrode. The water content was found by evaporating 1 ml. samples on a water-bath and drying overnight at 105° C. Freezing-point determinations were made by the apparatus and technique of Ramsay (1949).

Each estimation was made on a separate sample of blood taken from a single animal. All analytical results have been expressed in milligrams/gram (mg./g.) water. The calcium and carbon dioxide determinations were made on *Mytilus* which had been kept in sea water of a slightly different salinity from that in which the other *Mytilus* had been living. All *Mytilus* results have been standardized for an animal living in sea water containing 21·0 mg. Cl/g. water or 20·232‰ chlorinity.

III. THE COMPOSITION OF *MYTILUS* BLOOD

Details of the analyses are given in Table 1. Each value is the mean of at least four separate determinations. The composition of the sea water has been calculated from the chloride content using the ratios quoted by Sverdrup, Johnson & Fleming (1942).

Table 1. *Inorganic composition of* Mytilus *blood* (*mg./g. water*)

	Na	K	Ca	Mg	Cl	SO_4	CO_2	PO_4
Blood	11·54	0·497	0·505	1·357	20·78	2·944	0·220	0·0475
Sea water	11·66	0·420	0·445	1·406	21·00	2·932	0·111	—

Incomplete analyses of *Mytilus* blood have been published by Bethe & Berger (1931) and by Krogh (1939). For comparison, their results, re-calculated as ratios of the concentrations of ions in the blood to the concentration in the external medium, are given in Table 2.

Table 2. *Ratios of concentrations of ions in* Mytilus *blood to concentrations in sea water*

	Na	K	Ca	Mg	Cl	SO_4
Bethe & Berger (1931)	0·96	0·70	1·12	—	0·99	—
Krogh (1939)	0·995	1·75	—	—	0·94	0·875
Table 1	0·989	1·18	1·13	0·966	0·989	1·004

In sea water pH 8·0 the mean of eight measurements of the blood pH was 7·70. Individual results varied from 7·61 to 7·91. According to Cole (1940) the pH of the blood of the Lamellibranch *Venus* was 7·68 in sea water of pH 8·0. At a pH of 7·70 the sum of the cations in *Mytilus* blood is 651 m.equiv./kg. water and the sum of the anions 653. The difference is not significant.

Mytilus is very tolerant of brackish conditions. A comparison has been made of the freezing-point depressions of *Mytilus* blood and the external medium over a wide salinity range (Table 3). Dakin (1935) has reported that *Mytilus* blood is slightly hypertonic to sea water, but in the present series of measurements no significant difference was found.

Table 3. Mytilus edulis

Freezing-point depression of blood (° C.)	Freezing-point depression of sea water (° C.)
2·09	2·09
2·085	2·08
2·07	2·07
2·07	2·08
1·54	1·54
1·54	1·54
0·98	0·98
0·98	0·98
0·58	0·60
0·58	0·58

If allowance is made for water of crystallization, the dry weight of the blood not accounted for by the inorganic ions is only 1·6 mg./g. water. Two determinations of protein by Kjeldahl's method by Florkin & Blum (1934) were 1·2 and 1·7 mg./g. blood. Any contribution by organic substances to the osmotic pressure of the blood must be very small and therefore the state of ionization of the inorganic components of the blood must be very similar to that of sea water.

IV. THE COMPOSITION OF *ANODONTA* BLOOD

Since *Anodonta* shows considerable individual variation in composition the standard deviations are included in Table 4. Only a small portion of the standard deviations is considered to be due to experimental error.

Table 4. *Inorganic composition of* Anodonta *blood* (*mg./g. water*)

	Na	K	Ca	Mg	Cl	SO_4	CO_2	PO_4
Mean	0·358	0·0190	0·337	0·00465	0·415	0·073	0·643	0·0191
Standard deviation	0·015	0·0036	0·070	0·00047	0·036	0·017	0·135	0·0057
No. of determinations	4	5	17	4	14	6	14	12

Incomplete analyses of *Anodonta* blood have been published by Florkin & Duchâteau (1950). Their results re-calculated as mg./g. water content are: potassium 0·015, calcium 0·308, magnesium 0·006, chloride 0·420.

The mean of twenty determinations of the freezing-point depression of *Anodonta* blood is 0·0780° C. ± 0·009. As it is difficult to measure the freezing-point of distilled water to within less than 0·005° C. in the apparatus used, the results may suffer from a systematic error up to 0·005° C.

Schoffeniels (1951) has shown that 29 % of the calcium in *Anodonta* blood occurs in a non-ionized form. If allowance is made for this non-ionized fraction the remaining inorganic constituents of the blood would cause a freezing-point depression of 0·079° C.

The mean of twelve determinations of the pH of *Anodonta* blood was 7·52. Dotterweich & Elssner (1935) found the pH of the blood of *Anodonta* to be 7·29. At pH 7·52 the sum of the anions is 27·4 m.equiv./kg. water, and of the cations 28·4, allowing for the unionized calcium. The organic content of the blood, calculated from the dry weight and the inorganic content of the blood, is 0·76 mg./g. water.

V. THE SOLUBILITY OF CALCIUM CARBONATE IN LAMELLIBRANCH BLOOD

In Table 5 a comparison is made of the inorganic constituents of the blood of *Mytilus* and *Anodonta*. In view of the great difference in concentration it is interesting to note the similarity of the calcium and total carbon dioxide concentrations in the two animals. This suggests that both fluids may be saturated with respect to calcium carbonate.

Table 5. Mytilus edulis *and* Anodonta cygnea (*mg./g. water*)

	Na	K	Ca	Mg	Cl	SO_4	CO_2
Mytilus	11·54	0·497	0·505	1·357	20·78	2·944	0·220
Anodonta	0·358	0·0190	0·239*	0·00465	0·415	0·073	0·643
Mytilus / *Anodonta*	32·2	26·2	2·11	292·0	50·1	40·3	0·342

* Corrected for non-diffusible fraction.

To test this hypothesis it is necessary to compare the ionic products of calcium carbonate in the body fluids with the solubility products of calcium carbonate under the same conditions.

The concentration of carbonate ions can be calculated from the pH, and total carbon dioxide content of the blood and the apparent dissociation constants of carbonic acid. The dissociation constants vary with temperature and the ionic strength of the solution, but these variations have been investigated in detail by Buch, Harvey, Wattenberg & Gripenberg (1932) and Buch (1933).

The first apparent dissociation constant of carbonic acid K_1' is defined by the equation

$$K_1' = \frac{[H^+] \times [HCO_3^-]}{[H_2CO_3]}.$$

The second is defined by

$$K_2' = \frac{[H^+] \times [CO_3^=]}{[HCO_3^-]}.$$

The brackets indicate molar concentrations. The total carbon dioxide, as determined by Conway's method, is the sum of the carbonic acid, the bicarbonate and carbonate fractions.

For *Mytilus* at 10° C. and Cl 20·2 ‰.

$$K_1' = 0{\cdot}80 \times 10^{-6}. \quad (1)$$

$$K_2' = 0{\cdot}78 \times 10^{-9}. \quad (2)$$

Total carbon dioxide

$$(HCO_3^- + CO_3^{=} + H_2CO_3) = 5{\cdot}0 \text{ mM./kg. water.} \quad (3)$$

pH = 7·70, $H^+ = 2{\cdot}0 \times 10^{-8}$. Solving these three equations

$$HCO_3^- = 4{\cdot}70 \text{ mM} \quad \text{and} \quad CO_3^{=} = 0{\cdot}183 \text{ mM./kg. water.}$$

Anodonta blood has an ionic strength 0·035, equivalent to sea water of 0·95‰ chlorinity. At 12° C. 0·95‰ chlorinity, $K_1' = 4{\cdot}46 \times 10^{-7}$, $K_2' = 1{\cdot}25 \times 10^{-10}$, pH = 7·52. Therefore $H^+ = 3{\cdot}02 \times 10^{-8}$. Total $CO_2 = 14{\cdot}61$ mM./kg. water. Solving these equations as before

$$HCO_3^- = 13{\cdot}63 \text{ mM.} \quad \text{and} \quad CO_3^{=} = 0{\cdot}0562 \text{ mM./kg. water.}$$

The calcium concentration of *Mytilus* blood is 0·505 mg./g. water or 12·6 mM. Therefore the ionic product of calcium carbonate in *Mytilus* blood is

$$[Ca^{++}] \times [CO_3^{=}] = 0{\cdot}0126 \times 0{\cdot}000183 = 2{\cdot}31 \times 10^{-6}.$$

The concentration of diffusible calcium in *Anodonta* blood is 0·239 mg./g. water. If this is all ionized the ionic product of calcium carbonate in *Anodonta* blood is $0{\cdot}00596 \times 0{\cdot}0000562 = 0{\cdot}335 \times 10^{-6}$.

Calcium carbonate commonly occurs in two different crystalline forms, calcite and aragonite. Calcite is the more stable form and therefore is less soluble than aragonite, but aragonite is a common constituent of animal skeletons. The solubility of calcite in sea water has been investigated in detail by Wattenberg (1936) and Wattenberg & Timmermann (1936).

The solubility of aragonite in sea water was measured by Smith (1941). The solubility of an unspecified form of calcium carbonate in more dilute solutions was measured by Hastings, Murray & Sendroy (1926). Hastings *et al.* used an earlier series of values of K_1' and K_2' in their calculations so their relevant results have been re-calculated using Buch's figures (see Appendix). The temperature of the experiments, 38° C., would favour the precipitation of aragonite and the values obtained are in agreement with this. The results of the various determinations of the solubility product of calcium carbonate are given in Table 6.

Smith found that the solubility product of aragonite in sea water 36‰ salinity, at 30° C., was $1{\cdot}16 \times 10^{-6}$. If the temperature coefficient for aragonite is the same as that for calcite found by Wattenberg & Timmermann (1936), then the solubility product at 10° C. is $1{\cdot}95 \times 10^{-6}$. The ionic product of calcium carbonate in *Mytilus* blood, $2{\cdot}3 \times 10^{-6}$ is a little greater than this.

Similarly, the solubility product of aragonite at 12° C. in a solution equivalent in ionic strength to *Anodonta* blood is $0{\cdot}093 \times 10^{-6}$ and the ionic product is $0{\cdot}35 \times 10^{-6}$.

These results suggest that the blood of both animals is saturated or supersaturated with respect to aragonite. Other evidence is available which confirms this. When *Anodonta* blood is exposed to the air a thin film of calcium carbonate and protein is formed on the surface (De Waele, 1932). De Waele postulated that this was formed by the breakdown of a calcium protein complex with the loss of carbon dioxide. If the blood is saturated with calcium carbonate, calcium carbonate will be precipitated on exposure to air, as carbon dioxide is lost and the pH increased. Protein is commonly precipitated from solution in the presence of a precipitate of a bivalent ion.

Table 6. *The solubility product of calcium carbonate in sea water*

(1) Calcite 17·56‰ Cl (Wattenberg & Timmermann, 1936)

$T°$ C.	0	10	20	25	30
K_{CaCO_3}	0·83	0·74	0·62	0·52	$0·44 \times 10^{-6}$

(2) Calcite 20° C. (Wattenberg, 1936)

Salinity, ‰	0	5	10	25	35
K_{CaCO_3}	0·005	0·085	0·22	0·48	$0·62 \times 10^{-6}$

(3) Aragonite salinity 36‰, 30° C. (Smith, 1941)

$1·16 \times 10^{-6}$

(4) Calcium carbonate (see Appendix)

Ionic strength (μ) 0·031, 38° C., $pK\ CaCO_3 = 7·48$
0·050, 38° C., $pK\ CaCO_3 = 7·30$

Dotterweich & Elssner (1935) found that when the pH of the environment of *Anodonta* was artificially lowered, by keeping it in anaerobic conditions, the inner surface of the shell was visibly eroded and the blood calcium and carbon dioxide increased. Similar results were obtained by Dugal (1939) with *Venus mercenaria*. The pH of the blood recorded by Dotterweich & Elssner (7·29) was rather lower than that found by the writer (7·52), but the pH of the blood did not fall significantly during the course of their experiments. At this pH the blood would be just about saturated with respect to calcium carbonate.

VI. DISCUSSION

Hastings *et al.* (1926), after extensive investigations, demonstrated that human serum was in equilibrium with solid calcium carbonate and bone salts, although the apparent ionic products of calcium carbonate and calcium phosphate were greater than the solubility products in synthetic solutions of the same ionic strength. They concluded that some of the calcium was held in solution in unionized form. McLean & Hastings (1935) showed that approximately half the calcium was in the form of a calcium proteinate and considered that some of the rest was probably unionized citrate. Greenwald (1938) presented evidence that the calcium salts of some organic acids are largely unionized and so increase the apparent solubility product of calcium carbonate. But even if allowance is made for the unionized calcium some degree of supersaturation in vertebrate sera is indicated (Conway, 1945). In the Lamellibranchs and many other invertebrates the skeleton consists of

almost pure calcium carbonate and the protein content of the blood is much lower than in the vertebrates so the problem is simplified.

In Table 7 a comparison is made of the ionic products of calcium carbonate and the corresponding solubility products in a number of invertebrate sera. With the exception of *Anodonta* the calcium concentrations quoted are the total calcium concentration in the blood and probably include some unionized calcium. The highest degrees of apparent supersaturation are found in the two animals, *Homarus* and *Astacus*, with the highest blood protein concentrations. The fairly high supersaturation of *Anodonta* may well be spurious as the solubility product is widely extrapolated.

Table 7. *Ionic products of calcium carbonate and solubility products of aragonite in sea water and some animal sera*

Medium	μ	Ca (mM./l.)	pH	Total CO_2 (mM.^{2}/l.)	$K'_2 H_2CO_3 \times 10^9$	$CO_3^=$ (mM./l.)	$Ca \times CO_3 \times 10^6$	$K_{aragonite} \times 10^6$	Authorities for Ca, pH and CO_2
Sea water, deep ocean	0·73	10·5	8·10	2·50	0·63	0·181	1·90	2·1	Sverdrup *et al.* 1942; Smith, 1941
Mytilus	0·73	12·6	7·70	5·0	0·78	0·183	2·31	1·95	Potts
Asterias	0·62	11·2	7·37	2·50	0·76	0·0419	0·47	0·72 (calcite)	Cole, 1940
Limulus	0·60	16·0	7·47	6·2	0·75	0·13	2·08	1·74	Cole, 1940
Homarus	0·58	14·7	7·61	6·2	0·73	0·17	2·50	1·68	Robertson, 1949; Duval & Portier, 1927
Astacus	0·19	12·8	7·75	15·0	0·33	0·263	3·37	0·64	Duval & Portier, 1927; Bogucki, 1934
Helix	0·16	4·25	(7·5)	24·2	0·20	0·142	0·603	0·55	Florkin & Duchâteau, 1950; Duval & Portier, 1927
Anodonta	0·035	5·96	7·52	14·61	0·151	0·0562	0·335	0·093	Potts

With the exception of *Mytilus* (10° C.) and sea water (2° C.) all calculations have been made for 12° C. μ is the ionic strength of the solution.

The reported values of the pH of the sera of some animals differ according to various authors (e.g. Smith & Cole's determinations in Cole, 1940), but as far as possible the pH chosen has been that of the author of the inorganic analyses. In spite of the possible uncertainty of some of the pH values it is clear that there is a correlation between the solubility product of calcium carbonate and the ionic product of calcium and carbonate in the sera. In every case the ionic product is as great or greater than the solubility product. It is noteworthy that in *Asterias* the body fluid is nearly saturated with respect to calcite but not to aragonite. If allowance could be made for the unionized calcium and more accurate values of the pH of the sera and the solubility products of calcium carbonate were available, the agreement between columns eight and nine in Table 7 might well be closer.

The widespread occurrence of skeletons of calcium carbonate in marine animals must be correlated with the fact that sea water is normally supersaturated with

respect to calcium carbonate. The bulk of the ocean water of the world lies between 0 and 5° C. and is only slightly supersaturated but most of the surface waters of the world are at a higher temperature and are considerably supersaturated and will deposit calcium carbonate in the presence of suitable nuclei. The equatorial distribution of corals may be correlated with the greater degree of supersaturation in warmer waters.

The pH of the body fluids of marine animals usually lie in the range 7·4–7·8 and hence the carbonate fraction of the total carbon dioxide will be less than that in the sea water. As the calcium concentrations in the sera of marine animals are similar to the calcium concentration in sea water, the body fluids will be saturated with calcium carbonate if the total carbon dioxide in the body fluids is only 2–4 times as great as that of sea water, about 10–20 vol. % in the usual notation. The carbon dioxide content of the blood of marine invertebrates usually lies in this range.

The concentration of the blood of fresh water and terrestrial animals is usually much less (one-twentieth to one-third) than that of sea water. The solubility product of calcium carbonate in these solutions is very much lower than in sea water but this is balanced by the smaller degree of dissociation of carbonic acid in these solutions. In these conditions the body fluids will only be saturated with respect to calcium carbonate if the product of the calcium and total carbon dioxide concentrations is much greater than in marine species. In the fresh-water animals with calcareous skeletons for which data is available the calcium concentrations are less than that of sea water, but the total carbon dioxide concentrations are always very much greater, 30–60 vol. %. Duval & Portier (1927) commented, without explanation, on the very high carbon dioxide content of the bloods of fresh-water invertebrates.

Calcite is precipitated from pure supersaturated solutions of calcium carbonate at normal temperatures, but in the presence of small concentrations of strontium ions, such as occur in sea water, aragonite is precipitated instead. Above 30° C. aragonite is also precipitated from pure solutions but spontaneous precipitation of either form never occurs until the solution is very highly supersaturated. Deposition of either form can be obtained from only slightly supersaturated solutions if suitable nuclei are available (Wattenberg, 1936; Smith, 1941).

Little is known of the conditions which favour the deposition of calcite and aragonite respectively on different portions of the molluscan shell. Bevelander & Benzer (1948) observed the growth of calcite crystals in *Helix* on nuclei which apparently contained calcium and phosphate. A number of isomorphous substances, e.g. magnesium and manganese carbonates, can act as nuclei for calcite growth, and it is possible that the organic matrix may determine the type of crystal formed.

VII. SUMMARY

1. Analyses have been made of the inorganic constituents of the blood of *Mytilus edulis* and *Anodonta cygnea*.

2. *Mytilus* blood resembles sea water in total concentration and composition, but has greater concentrations of calcium, potassium and total carbon dioxide.

3. *Anodonta* blood has a very low total concentration of solutes, but the concentrations of calcium and carbon dioxide are of the same order of magnitude as the concentrations in *Mytilus* blood.

4. This is discussed with reference to solubility of calcium carbonate in ionic solutions, and it is concluded that the blood of both animals is saturated with respect to aragonite.

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VIII. APPENDIX

Solubility product of calcium carbonate in very dilute solutions.

Hastings *et al.* (1926, p. 746) provide the following information:

Exp. 2. $\mu = 0{\cdot}031$, pH 7·22, total $CO_2 = 24{\cdot}56 \times 10^{-3}$ mole, pCa = 3·43, 38° C.

$\mu = 0{\cdot}031$ is equivalent to sea water 0·833‰ Cl.

From Buch *et al.* (1932), at 38° C. and 0·833‰ Cl

$$pK_1' = 6{\cdot}185. \qquad (1)$$

From Buch (1933), at 38° C. and 0·833‰ Cl

$$pK_2' = 9{\cdot}627, \qquad (2)$$

$$(H_2CO_3 + HCO_3^- + CO_3^{=}) = 24{\cdot}56 \times 10^{-3}. \qquad (3)$$

Solving these three equations:

$$CO_3 = 8{\cdot}925 \times 10^{-5},$$

$$pCO_3 = 4{\cdot}0494,$$

therefore $pK\ CaCO_3 = 7{\cdot}479$.

Exp. 3. $\mu = 0{\cdot}050$, pH = 7·07, total $CO_2 = 18{\cdot}93 \times 10^{-3}$ mole, pCa = 3·06.

$\mu = 0{\cdot}050$ is equivalent to sea water 1·344‰ Cl.

As before

$$pK_1' \text{ (38° C., 1·344‰ Cl)} = 6{\cdot}155, \qquad (1)$$

$$pK_2' \text{ (38° C., 1·344‰ Cl)} = 9{\cdot}525, \qquad (2)$$

$$CO_2 + HCO_3^- + CO_3^{=} = 18{\cdot}93 \times 10^{-3} \text{ mole}; \qquad (3)$$

therefore

$$CO_3 = 5{\cdot}775 \times 10^{-5},$$

$$pCO_3^{=} = 4{\cdot}2385,$$

therefore $pK\ CaCO_3 = 7{\cdot}298$.

If $pK\ CaCO_3 = 7{\cdot}298$ when $\mu = 0{\cdot}050$ and 7·479 when $\mu = 0{\cdot}031$ then by interpolation when $\mu = 0{\cdot}035$ $pK\ CaCO_3 = 7{\cdot}441$. Therefore

$$K\ CaCO_3 = 3{\cdot}62 \times 10^{-8} \text{ at } 38° \text{ C.}$$

From Wattenberg & Timmermann's figures (1936) K (calcite, 17·56‰ Cl, 12° C.) is $0{\cdot}72 \times 10^{-6}$ and, by extrapolation, K (calcite, 17·58‰ Cl, 38° C.) is $0{\cdot}28 \times 10^{-6}$. Assuming the same temperature coefficient for aragonite, the solubility product of aragonite in a solution $\mu = 0{\cdot}035$, 12° C., is $0{\cdot}93 \times 10^{-7}$.

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THE EXCHANGE OF LABELLED SODIUM IN THE LARVA OF *AEDES AEGYPTI* L.

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INTRODUCTION

The osmotic and ionic regulation of mosquito larvae has been the subject of a number of investigations during the last twenty years. Wigglesworth (1933*a*, *b*) showed that while the general body surface of the larva of *Aedes aegypti* was relatively impermeable to salts and water, the anal papillae were permeable to both these substances. Later, Wigglesworth (1938) was able to show that mosquito larvae could regulate the osmotic pressure and chloride content of the haemolymph in the face of quite large variations in the concentration of the external medium. The work of Koch (1938) demonstrated the ability of larvae to take up chloride ions from the external medium against steep concentration gradients, while the recent work of Ramsay (1953) has established that the papillae also take up sodium and potassium from dilute solutions. Ramsay (1951) has investigated the part played by the Malpighian tubules in sodium excretion. He found that the tubules were capable of excreting fluid containing appreciably less sodium than the haemolymph. The concentration of potassium, on the other hand, was found to be higher in the tubules than in the haemolymph (Ramsay, 1953). There seems to be reabsorption of both these elements into the haemolymph by the rectum. Ramsay also gives figures which indicate that the larva of *A. aegypti* is able to regulate the concentration of sodium and potassium in the haemolymph when the external concentrations are varied.

Previous work on ionic exchange in mosquito larvae has been concerned with the loss, or entry, of ions produced by large differences in concentration between the haemolymph and the external medium. So far no work has been done with radioactive isotopes on the rate of exchange of ions between the haemolymph and the external medium. The object of this study was, therefore, to investigate the exchange of labelled sodium between the haemolymph and the external medium, and, if possible, to determine the rate at which this process occurred. It was hoped that by suitably altering certain of the external conditions it would be possible to throw some light on the nature of the mechanisms involved.

THEORETICAL CONSIDERATIONS

When a cell is placed in a solution containing ^{23}Na and ^{24}Na, the ^{23}Na within the cell will exchange with both isotopes of sodium outside. The entry of ^{24}Na may be used as a measure of the total sodium entering the cell, the mixed population of ^{23}Na and ^{24}Na being labelled by the proportion of ^{24}Na which it contains. The increase in the concentration of the labelled sodium within the cell should proceed

so that equilibrium is achieved asymptotically. If all the cell sodium is exchangeable then at infinite time the concentration of the labelled population of sodium ions should be equal to the total sodium content of the cell, as determined by chemical analysis at finite time. This is true only if the total sodium content and the volume of the cell remain constant during the experiment. ^{23}Na is chemically identical with its unstable isotope ^{24}Na and it is assumed that cells do not distinguish between these two isotopes.

Equations have been worked out (e.g. Harris & Burn, 1949; Davson, 1951) from which the transfer constants for ionic penetration may be calculated. The symbols used here are as follows:

k_{in} = transfer constant in the direction out to in.

k_{out} = transfer constant in the direction in to out.

t = time, in hours.

$[Na_{in}]_\infty$ = internal concentration of labelled sodium at infinite time (being derivable from total cell Na at finite time).

$[Na_{out}]$ = external concentration of labelled sodium.

$[Na_{in}]t$ = concentration of labelled sodium within the cell at any time t.

The transfer constants are related to the concentration of Na in the cell and the external medium by the formula

$$k_{in} = k_{out}\,[Na_{in}]_\infty\,[Na_{out}]. \tag{1}$$

Following conventional methods the cell is placed in a solution of labelled sodium and $[Na_{in}]t$ is determined at intervals of time. Using the equation

$$-k_{out}\,t = \ln\left(1 - \frac{[Na_{in}]t}{[Na_{in}]_\infty}\right), \tag{2}$$

$\ln\left(1 - \dfrac{[Na_{in}]t}{[Na_{in}]_\infty}\right)$ is plotted against time and the slope of the straight line gives the value of k_{out}.

In a semi-logarithmic plot of equation (2) above, the variation increases with time, for, as Solomon (1952) has pointed out, as $[Na_{in}]t$ approaches $[Na_{in}]_\infty$ the error becomes progressively larger, so that only the first few points accurately describe the straight line. It is not permissible, therefore, to calculate a regression line to obtain the slope of the line. In each case the line drawn through the points is the mean of the slopes calculated from the lines joining the individual readings to the point of origin.

The equations, outlined above, require that $[Na_{in}]_\infty$ be known independently of the experiment. If the original data of $[Na_{in}]t$ as a function of time is of sufficient quality for differentiation it is possible to use the following equation

$$\frac{d[Na_{in}]t}{dt} = k_{out}\,([Na_{in}]_\infty - [Na_{in}]t), \tag{3}$$

whereby, plotting $\frac{d[Na_{in}]t}{dt}$ against $[Na_{in}]t$, both $[Na_{in}]_\infty$ and k_{out} can be determined from the graph.

The constants k_{in} and k_{out}, used above, are independent of cell volume and surface area, having the units l./time (hours^{-1}).

The equations governing the rate of exchange of radioactive ions have been worked out chiefly for single cell systems, such as the erythrocyte, although similar equations have been used to describe the uptake of labelled sodium by *Daphnia magna* (Holm-Jensen, 1948). The application of such equations to a complex system such as a mosquito larva must be done with caution and with due regard to the limitations involved. The exchange of sodium between the haemolymph and the external medium is complicated by the presence of various tissue compartments and by the fact that the exchange of sodium does not occur across a single membrane. The sodium is taken up chiefly by the anal papillae and lost in the dilute urine passing out of the rectum (Ramsay, 1953). There is reason to suppose that the sodium content of the tissues is low (Ramsay, 1953), so that the exchange between the tissues and the haemolymph might not significantly affect the exchange between the haemolymph and the external medium. The plot of $\ln\left(1-\frac{[Na_{in}]t}{[Na_{in}]_\infty}\right)$ against time should result in a straight line if the tissue compartments are not a significant factor in the haemolymph sodium exchange. If the tissue compartments are a significant factor then the data should not fit, and the semi-logarithmic plot should result in a curve. It will be seen from Fig. 2 that the plot results in a fairly straight line; it is therefore assumed that the tissue compartments do not significantly affect the exchange between the haemolymph and the external medium, and that the system can, as an approximation, be considered as having two compartments only. The value of $[Na_{in}]_\infty$ used in plotting these results was taken as 100 m.equiv./l. which is the figure given by Ramsay (1953) for larvae kept at a low external sodium concentration of 1·7 m.equiv./l. The experiments to be described were carried out at a slightly higher concentration, chiefly at 4·00 m.equiv./l. As the concentration of the sodium in the haemolymph remains relatively constant in the face of large differences in the external concentration, being 113 m.equiv./l. at an external concentration of 85 m.equiv./l. (Ramsay, 1953), it is assumed that the internal sodium level will differ but little over the range of concentrations used in these experiments. This is supported by the fact that the line for the plot of $\frac{d[Na_{in}]t}{dt}$ against $[Na_{in}]t$ cuts the abscissa at a value close to 100 m.equiv./l. (Fig. 3).

The fact that the exchange of sodium in the mosquito larva does not occur across one membrane, but across a number, limits the use of the transfer constants. k_{in} and k_{out} are used as a resultant index of the sodium turnover in the whole system. k_{in} may be described as a measure of the proportion of the outside concentration taken up in unit time; similarly, k_{out} is a measure of the proportion of the inside concentration lost in unit time.

MATERIALS AND METHODS

The larvae of *Aedes aegypti* were reared from eggs in a balanced salt solution at a temperature of 28° C. They were fed on a mixture of 'Bemax' and powdered dog biscuit. On reaching the fourth instar they were transferred to a medium chemically similar, but without food in it. After 3 days in this they were transferred to a medium, again chemically the same, in which a very small part of the sodium was replaced by the radioactive isotope ^{24}Na.

The composition of the balanced salt solution was as follows:

	mM./l.		mM./l.
NaCl	4·00–8·00	KH_2PO_4	0·10
$MgCl_2$	0·20	KOH	0·059
$CaCl_2$	0·50		

The KH_2PO_4–KOH buffer was used to raise the pH of the solution to 6·3.

The ^{24}Na was obtained from Harwell in the form of Na_2CO_3. It was converted to NaCl by adding an equivalent amount of HCl and heating until dry. Two further evaporations were carried out, after the addition of distilled water, to drive off any excess HCl.

The counting of radioactive samples was carried out with a G.E.C. G.M. 2 type end-window counter and a Dynatron scaler unit. Samples were pipetted on to counting disks and dried before counting. The counting rate fell progressively during each experiment as the ^{24}Na decayed, and the counting error therefore increased. For the greater part of an experiment counts were made with a standard deviation of less than 3 %, and on the lowest counts the counting time was extended so that the standard deviation due to the counting error did not rise above 5 %. Appropriate dead-time corrections were made whenever necessary.

The decay of ^{24}Na was followed, in each experiment, over a period of about 100 hr. The half-life obtained was $14{\cdot}88 \pm 0{\cdot}10$ hr., which was very close to the Harwell estimate of 14·9 hr. It should be mentioned that estimates for the half-life of ^{24}Na have been given which range from 14·8 hr. (Van Voorhis, 1936) to 15·10 hr. (Cobble & Atteberry, 1950). A small difference in the estimate of the half-life of short-lived isotopes will produce quite large differences towards the end of an experiment, which is one of the disadvantages of using isotopes such as ^{24}Na and ^{42}K.

In order to count the radioactivity of the larvae it was necessary to remove ^{24}Na from the body surface. To do this the larvae were placed in a short glass tube closed at one end by a piece of bolting silk, and were washed in a slow stream of tap water. It was found, by washing larvae which had been quickly dipped in a radioactive sodium solution, that all the ^{24}Na was removed within 3 min. It is assumed that only a negligible amount of ^{24}Na will be lost from the haemolymph in this time.

To collect the haemolymph the larvae were first dried on a filter-paper, and then punctured with a fine needle on a slide coated with paraffin wax. The haemolymph was then rapidly drawn into silicone-lined pipettes and transferred to counting disks. In the early stages of an experiment the volume of haemolymph collected was 0·2–0·3 $mm.^3$. As the activity of the ^{24}Na became weaker larger pipettes were

used, with volumes of up to 1·2 mm.^{3}, and the haemolymph collected from several larvae. It was noticed that the haemolymph tended to coat the inside of the pipettes fairly rapidly, so as to reduce the volume slightly, and therefore a number of pipettes were used in each experiment. The pipettes were calibrated by counting the activity of the number of pipettes filled with radioactive solutions of known concentration. The activity of the ^{24}Na in the haemolymph is expressed as 1000 counts/mm.^{3}/min. (corrected to zero time).

To determine the radioactivity of the whole body five to ten larvae were dried on a filter-paper and weighed on a balance accurate to 0·01 mg. They were then homogenized in 1·0–4·0 ml. of distilled water in a tissue homogenizer, the volume of water and the number of larvae depending on the activity of the sodium. In each case two 0·2 ml. samples of the homogenate were then taken and pipetted on to counting disks, dried in an oven at 120° C. and later counted. The mean of the two samples was taken and the activity is expressed as 1000 counts/mg. tissue/min., all counts being corrected to zero time.

All experiments were carried out at a temperature of 28° C. ($\pm$0·2° C.) unless it is stated otherwise. The experimental solutions used in each case were well aerated with compressed air.

RESULTS

The uptake of labelled sodium

Larvae, reared as previously described, were placed in a balanced salt solution containing 4·00 m.equiv./l. sodium labelled with ^{24}Na. The uptake of sodium into the haemolymph and into the whole body was followed over a period of 120 hr.

The results obtained are illustrated in Fig. 1. Each point on the graph for the haemolymph represents the mean of five determinations, the vertical lines show the extent of the standard error. In the graph showing the increase in the sodium concentration of the whole body single readings have been plotted. It will be seen that at the end of the experiment the concentration of labelled sodium in the haemolymph had risen to a level of approximately 70 m.equiv./l.; while for the whole body the level was equivalent to 0·060 m.equiv./g. tissue (wet weight). These results show that the haemolymph sodium exchanges with that in the outside medium. Using the figure of 100 m.equiv./l. for the concentration of sodium in the haemolymph (Ramsay, 1953), the time taken for half the total sodium to exchange is of the order of 62 hr.

It appears from the two figures in Fig. 1 that the uptake of labelled sodium into the haemolymph can be represented as parallel to the uptake by the whole body. This may be due to the fact that the exchange of sodium between the haemolymph and the tissues is small compared with the exchange between the haemolymph and the external medium.

Using equation (2) above, a semi-logarithmic plot results in a fairly satisfactory straight line (Fig. 2). The line shown in Fig. 2 is the mean of the slopes calculated from the lines joining the individual readings to the point of origin. Using a value of $[Na_{in}]_\infty = 100$ m.equiv./l., $k_{out} = 0{\cdot}011$ hr.$^{-1}$ and $k_{in} = 0{\cdot}276$ hr.$^{-1}$.

The results for the uptake of sodium, at 28° C., illustrated in Fig. 8, appeared to show least variation and were used to plot $\frac{d[Na_{in}]t}{dt}$ against $[Na_{in}]t$. The differentials with respect to time were calculated from a curve drawn through the points (solid circles in Fig. 8). This method, though necessarily rather crude, shows that a

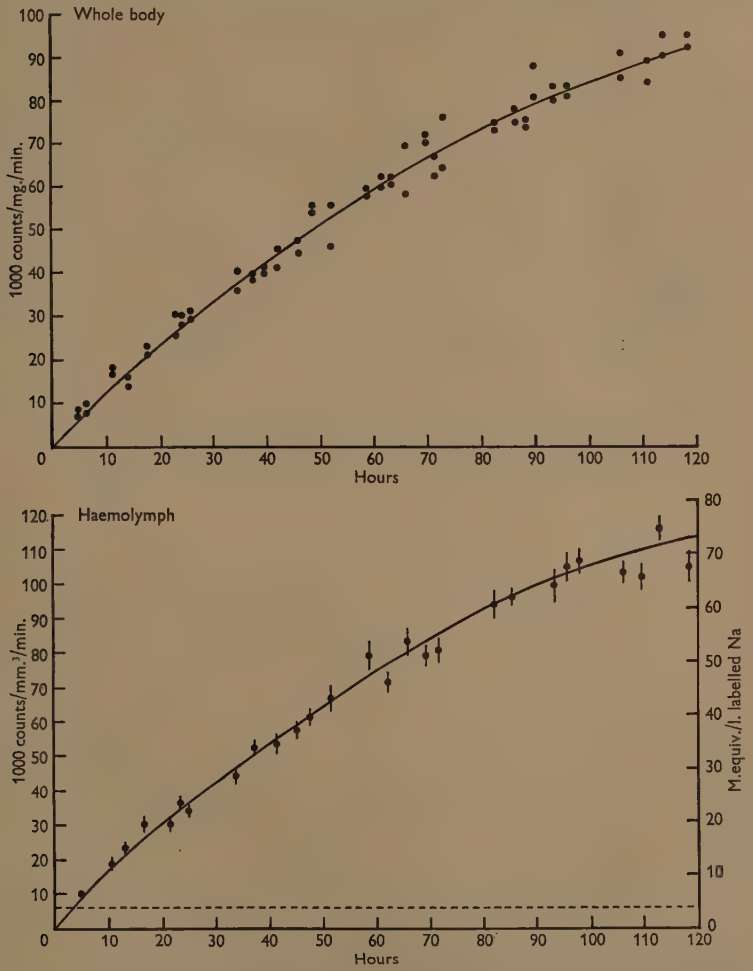

Fig. 1. The uptake of labelled sodium into the whole body and haemolymph. The ordinate on the left for the haemolymph is the activity of the ^{24}Na expressed as 1000 counts/mm.^{3}/min.; the one on the right being the concentration of labelled sodium expressed as m.equiv./l. Na. The broken line indicates the concentration of labelled sodium in the external medium.

straight line (Fig. 3) can be obtained from a curve which would appear to fit the data rather well (Fig. 8). The line cuts the abscissa at a value of 103 m.equiv./l., which approximates to the figure of 100 m.equiv./l. for the sodium concentration of the haemolymph given by Ramsay (1953).

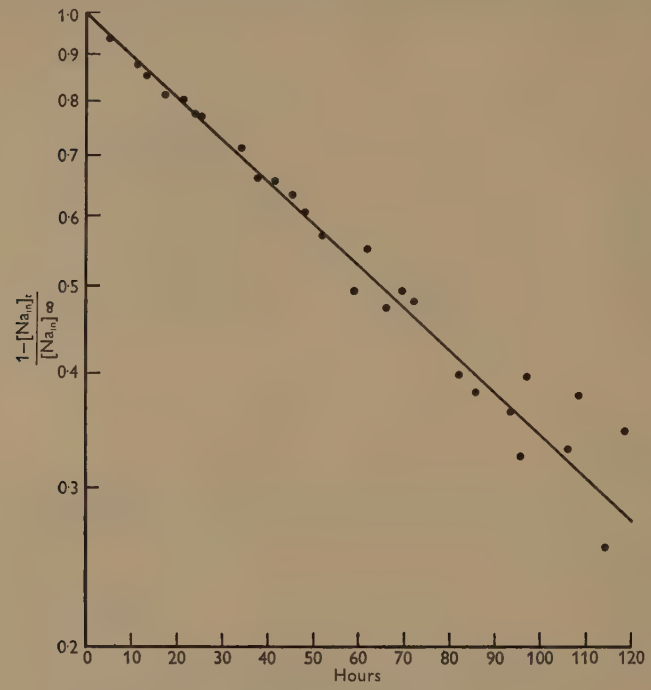

Fig. 2. The data for the haemolymph, illustrated in Fig. 1, plotted according to equation (2).

Site of uptake of sodium

To determine the site of uptake of sodium the entry of ^{24}Na into the haemolymph was compared as between: (i) normal larvae; (ii) larvae with anal papillae destroyed; (iii) larvae with anal papillae destroyed and mouths blocked.

The papillae were destroyed by immersing larvae in 5% NaCl for 5 min. and then returning them to a normal balanced salt solution (Wigglesworth, 1933*b*). The mouth was blocked by placing a small drop of Whitehead's varnish on the mouthparts. This prevented the entry of fluid into the gut, for when such larvae were placed in a solution of Trypan Blue there was no sign of the dye in the gut, while in normal larvae the gut quickly filled with the dye.

From Fig. 4 it will be seen that the uptake of labelled sodium occurred much more rapidly in normal larvae than in those with no anal papillae. In the larvae with their

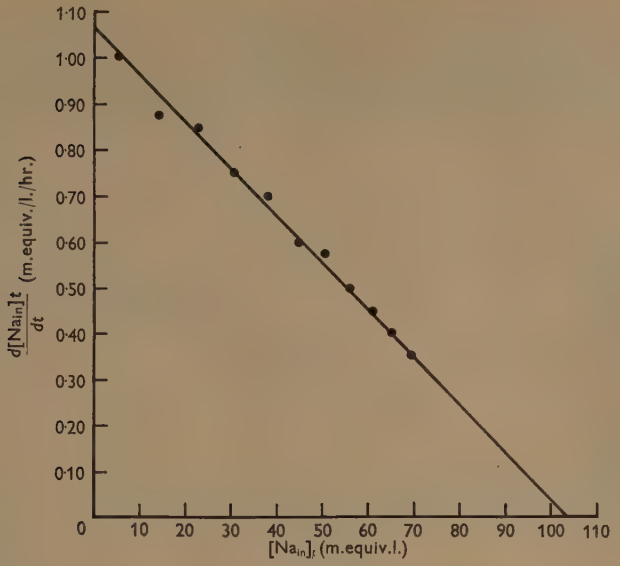

Fig. 3. The data illustrated in Fig. 8 (28° C.) plotted according to equation (3).

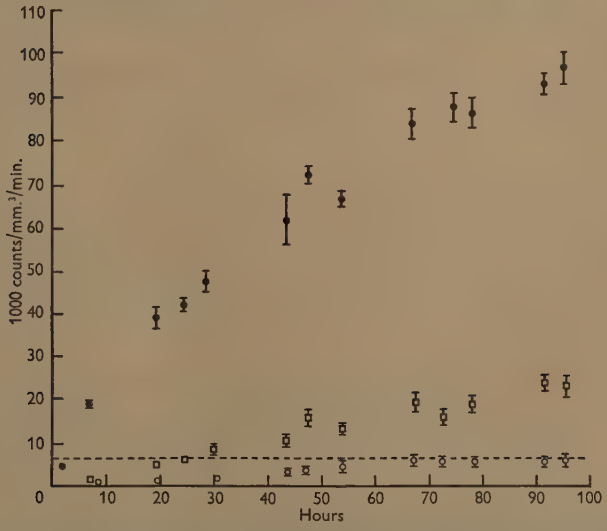

Fig. 4. The uptake of labelled sodium into the haemolymph of normal larvae (solid circles), larvae with the anal papillae destroyed (open squares) and larvae with the papillae destroyed and mouths blocked (open circles). The broken line indicates the concentration of labelled sodium in the external medium.

mouths blocked and the anal papillae destroyed there was only a very slow entry into the haemolymph, for after 96 hr. the concentration of ^{24}Na in the haemolymph was no greater than that in the external medium. It is clear, therefore, that most of the sodium enters the haemolymph via the anal papillae, while a much smaller proportion enters through the gut, the body surface being relatively impermeable to sodium.

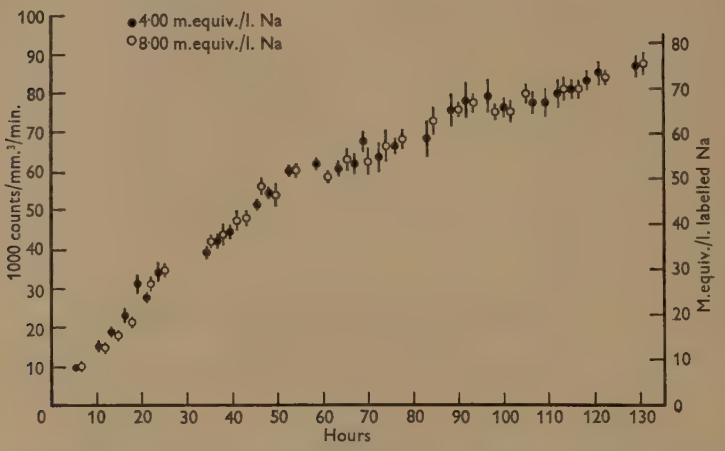

Fig. 5. The effect of concentration on the uptake of labelled sodium into the haemolymph. Concentration of sodium in the external media: 4·00 m.equiv./l. (solid circles) and 8·00 m.equiv./l. (open circles).

The effect of external concentration of sodium

Two lots of larvae were reared in solutions of 4·00 m.equiv./l. and 8·00 m.equiv./l. NaCl and were then transferred to similar solutions containing labelled sodium. The uptake of labelled sodium into the haemolymph from both concentrations of external sodium is shown in Fig. 5. Both series of points appear to follow the same curve, and it would seem therefore that the uptake of sodium proceeded at a similar rate in the two solutions.

From the work of Ramsay it is known that the concentration of sodium in the haemolymph remains relatively constant over a fairly wide range of external sodium concentrations. It is assumed, therefore, that the internal concentration of sodium will not vary significantly between 4·00 and 8·00 m.equiv./l. of external sodium. Using a value of $[Na_{in}]_{\infty} = 100$ m.equiv./l., a semi-logarithmic plot of the function of concentration against time results in an approximately straight line in both cases (Fig. 6). The resultant slopes give values for k_{out} of 0·012 hr.$^{-1}$ for both the 8·00 and the 4·00 m.equiv./l. solutions. This results in a value of $k_{in} = 0{\cdot}15$ hr.$^{-1}$ for the 8·00 m.equiv./l. solution and $k_{in} = 0{\cdot}30$ hr.$^{-1}$ for the 4·00 m.equiv./l. solution.

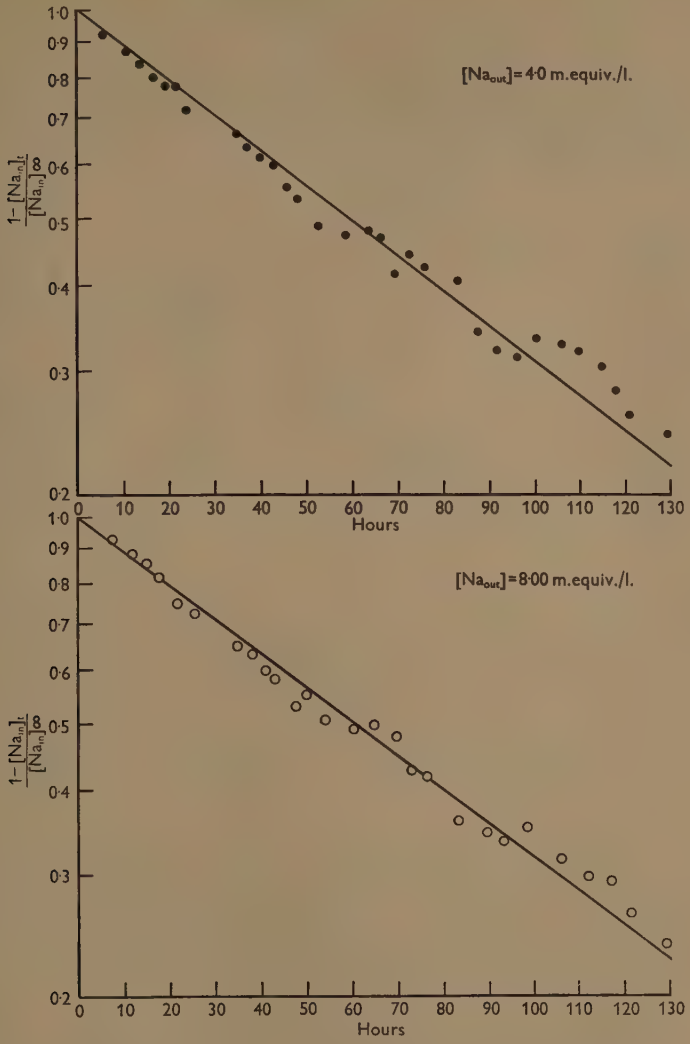

Fig. 6. The data illustrated in Fig. 5 plotted according to equation (2).

The effect of external potassium

To study the effect of external potassium on the entry of sodium into the haemolymph, one batch of larvae was placed in a solution containing 4·00 m.equiv./l. of labelled sodium with the usual concentration of potassium (0·159 m.equiv./l.),

while a second batch was placed in a solution in which the potassium concentration was increased to 4·00 m.equiv./l. by the addition of KCl.

From Fig. 7 it will be seen that the increase in the concentration of the external potassium had no significant effect on the rate of uptake of ^{24}Na, even though the potassium concentration was equal to that of the sodium in one case. It seems clear, therefore, that potassium ions do not compete with sodium for uptake into the haemolymph.

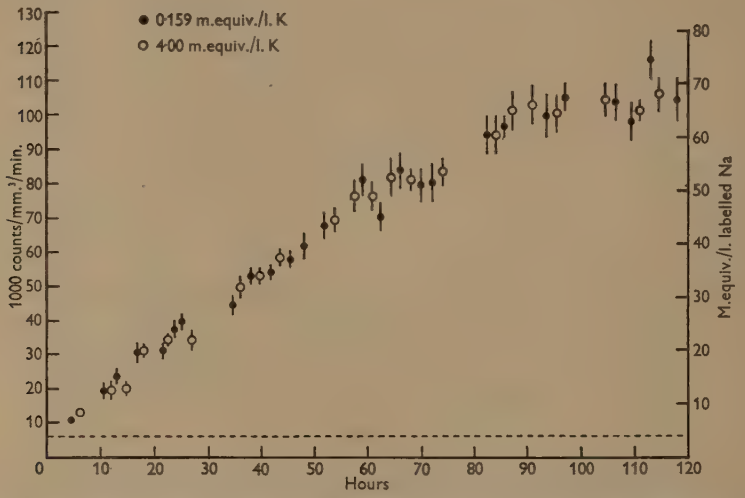

Fig. 7. The effect of external concentration of potassium on the uptake of labelled sodium into the haemolymph. Concentrations of potassium in the external media: 0·159 m.equiv./l. (solid circles) and 4·00 m.equiv./l. (open circles). The broken line represents the concentration of labelled sodium in the external media.

The effect of temperature

The uptake of labelled sodium from a 4·00 m.equiv./l. solution of NaCl was investigated at three temperatures—20, 28 and 35° C. The larvae, which were reared from the eggs at a temperature of 28° C., were kept at the experimental temperature for 3 days before being placed in the labelled sodium solutions.

The results, shown in Fig. 8, indicate that the rate of uptake was distinctly more rapid at 35° C. than at 28° C. and slightly slower at 20° C. Thus an 8° C. rise from 20 to 28° C. has less effect than a 7° C. rise from 28 to 35° C.

It is possible that the concentration of sodium in the haemolymph (i.e. $[Na_{in}]_\infty$) may vary with temperature. In the absence of any evidence to the contrary it would seem unwise to calculate any transfer constants, which might give misleading results. The results for 20 and 35° C. are not of sufficient quality for plotting $\frac{d[Na_{in}]t}{dt}$ against concentration to obtain k values.

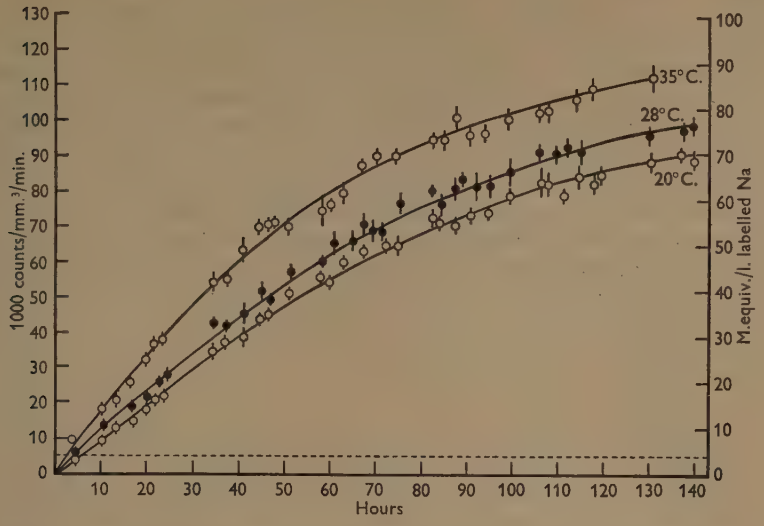

Fig. 8. The effect of temperature on the uptake of labelled sodium into the haemolymph.

The effects of distilled water

This experiment was designed to study the effect of washing in distilled water on the rate of loss of labelled sodium from the haemolymph. Larvae were placed in a 4·00 m.equiv./l. solution of labelled sodium and after a period of 48 hr. they were placed in a slow current of glass-distilled water. The activity in the haemolymph was very low towards the end of the experiment, with a consequent increase in the counting error.

The results are summarized in Fig. 9; each point is the mean of five readings, the standard error being indicated by the vertical lines. It will be seen that after an initial fall there is only a very slow decrease in the sodium level over a period of 80 hr. The mosquito larva is capable, therefore, of retaining its sodium, for a time at least, in the face of an extreme concentration gradient.

Tests carried out during the experiment showed that there was no detectable radioactivity present in the distilled water, and it seems unlikely that the larvae were able to reabsorb the sodium which was lost from the haemolymph.

DISCUSSION

The results described above are in agreement with the observations of Ramsay (1953) that sodium ions are absorbed from dilute solutions by the anal papillae, for most of the exchange of labelled sodium occurred through the papillae. In addition, however, it appears that smaller proportions enter the haemolymph via the gut and general body surface. The papillae are also capable of secreting chloride ions into the haemolymph against a concentration gradient (Koch, 1938). Similar organs in

the larva of the beetle *Helodes* actively absorb chloride, although in this case a higher proportion is taken up through the gut (Treherne, 1954).

The fact that the data for the uptake of labelled sodium into the haemolymph seems to fit the theoretical relationship for a two-compartment system suggests that most of the sodium exchange occurs between the haemolymph and the external medium rather than between the haemolymph and the tissues. This is also supported by the fact that the uptake of labelled sodium into the haemolymph and the whole body can be represented as similar smooth curves. Now the rate of entry of labelled sodium into the haemolymph should proceed most rapidly at the beginning, while, as the concentration in the haemolymph is steadily rising, it is clear that the rate of entry into the tissues will be at maximum at some time later. Therefore,

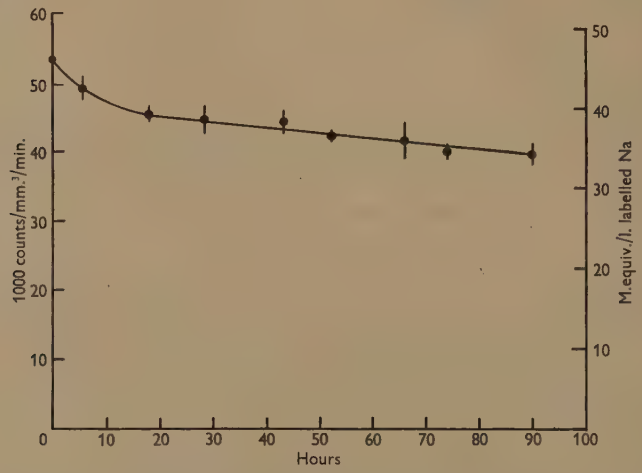

Fig. 9. The effect of starvation in flowing glass-distilled water on the loss of sodium from the haemolymph of larvae previously kept in a 4·00 m.equiv./l. solution of labelled sodium for 48 hr.

theoretically, the increase of concentration in the tissues should proceed as a sigmoid curve. If there is a significant exchange between the haemolymph and the tissues, then the curve for the uptake of labelled sodium by the whole body should be of a different shape from that for uptake into the haemolymph alone. As there appears to be little difference between the two curves it is assumed that any exchange between the tissues and the haemolymph is small compared with that between the haemolymph and the external medium. This is perhaps to be expected if the tissue sodium content is low, which would seem to be the case from the observations of Ramsay (1953).

From the line obtained when $\frac{d[Na_{in}]t}{dt}$ is plotted against concentration (Fig. 3) it was found that approximately 103 m.equiv./l. Na are exchangeable with the

sodium in the outside medium. This is very close to the figure of 100 m.equiv./l. given by Ramsay (1953), which was obtained by direct chemical analysis of the haemolymph, and suggests that all the haemolymph sodium is exchangeable; it is in any case unlikely that any sodium would be present in an unionized or 'bound' form.

The transfer constant k_{in} was found to be greater than k_{out} by a factor depending on the concentration difference between the haemolymph and the external medium. This differential permeability is probably the result of an active transport mechanism, either the cation or the anion being actively transported. It should be mentioned, however, that this process cannot be strictly defined as active transport without some knowledge of the electrical potential across the membrane (Ussing, 1949).

The rates of uptake of labelled sodium were shown to be similar at concentrations of 4·00 and 8·00 m.equiv./l. NaCl. The calculated values for k_{out} were similar at both concentrations, but the k_{in} values differed by a factor of approximately 2. This means that the larvae take up differing proportions of sodium from the external medium at different concentrations, in such a way that a constant rate of uptake is maintained. Thus it would seem that in these experiments the rate of uptake was independent of the external concentration. This implies that, at these concentrations at least, the uptake mechanism plays a part in maintaining a relatively constant internal concentration of sodium. It would perhaps be desirable to repeat these experiments over a wider range of external concentrations, determining the level of haemolymph sodium by the flame photometer method (Ramsay, Brown & Falloon, 1953). It is known that at higher external concentrations the Malpighian tubules can assist in regulation by excreting a fluid having a higher concentration of sodium (Ramsay, 1953), although there is no evidence that they can excrete a fluid containing more sodium than the haemolymph.

Larvae kept in a current of glass-distilled water were able to retain the sodium in the haemolymph, after an initial drop, with only a slight loss over 80 hr. It seems unlikely that the level of sodium in the haemolymph is being maintained by a leakage from the tissues into the haemolymph, for the tissue sodium is probably much lower than that in the haemolymph. In this case the conservation must be effected by the Malpighian tubules, which have been shown to excrete a fluid containing little sodium when the larva is kept in distilled water (Ramsay, 1951), and by reabsorption by the rectum.

Wigglesworth (1938) showed that chloride was lost fairly rapidly from the haemolymph of mosquito larvae kept in distilled water. The results using labelled sodium and those involving direct chemical analysis (Ramsay, 1953) indicate that the sodium ion is not lost so rapidly from the haemolymph. It is possible that the larva behaves differently with respect to the two ions, retaining the sodium more effectively than the chloride. One explanation of this might be that the chloride ions pass out with excreted divalent cations, thus producing a more rapid loss to the exterior. Alternatively, it may be that anion and cation absorption are independent, as in *Eriocheir* (Krogh, 1938).

It has been shown that alteration of the external potassium concentration does

not affect the rate of uptake of sodium into the haemolymph. Potassium ions do not, therefore, compete with sodium ions for transport into the haemolymph. The fact that potassium is absorbed, chiefly by the anal papillae, against a concentration gradient (Ramsay, 1953) suggests that sodium and potassium are transported into the haemolymph by different mechanisms. Specific mechanisms, capable of separating Na^+ and K^+, have been demonstrated in some other animals; for example Krogh (1937) showed that while NaCl is actively absorbed by the frog skin, from dilute solutions of NaCl, only Cl^- is absorbed from KCl solutions. Little appears to be known about the workings of a system capable of separating two such closely related cations.

SUMMARY

1. The exchange of labelled sodium between the external medium and the haemolymph and whole body has been investigated in the larva of *Aedes aegypti*. The time for half exchange was of the order of 62 hr.

2. It was found that most of the exchange of labelled sodium occurred through the anal papillae, although smaller amounts enter the haemolymph through the gut and general body surface. Transfer constants have been used to describe the resultant turnover of labelled sodium in the whole system.

3. The rate of uptake of sodium was independent of the external concentrations used in these experiments.

4. Potassium ions do not compete with sodium for uptake, which suggests that separate mechanisms are responsible for the accumulation of these two ions.

5. Larvae were able to retain the sodium in the haemolymph, with relatively little loss, in glass-distilled water.

6. The effect of temperature on the rate of uptake of labelled sodium has been investigated.

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OSMOTIC RESPONSES IN THE SIPUNCULID *DENDROSTOMUM ZOSTERICOLUM*

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INTRODUCTION

Members of the Phylum Sipunculoidea are generally poikilosmotic animals which inhabit intertidal regions and subtidal zones of the sea where salinities rarely change. Several token investigations have been conducted on the osmotic behaviour of this group, particularly with the genus *Sipunculus* (Quinton, 1900; Schücking, 1902; Dekhuyzen, 1921; Bethe, 1934). Adolph (1936), however, performed an extensive study of the sipunculid *Phascolosoma gouldi*, which he described as behaving like an osmometer when subjected to osmotic stresses, i.e. it seemed to be impermeable to salts. A like behaviour was suggested by Dekhuyzen (1921) for *Sipunculus* and for *Phascolosoma vulgare*. However, these conclusions were based on rates of weight change in given osmotic stresses assuming that the number of osmotically active particles in the internal fluid remain constant and that the gut and nephridia do not influence the observed phenomena. They found no evidence of volume control, i.e. the ability to return toward normal size when experimentally forced away from it in dilute or concentrated sea water. Adolph (1936) did show a tendency for *Phascolosoma* to regain weight when the blood volume was experimentally reduced. On the other hand, Koller (1939) showed that *Physcosoma japonicum* controls its volume when immersed in dilutions as high as 65% sea water, but does not return toward normal volume after prolonged immersion in 50% sea water.

In the present investigation it is shown that the sipunculid *Dendrostomum* behaves superficially as an osmometer, but it is actually more complex because: (*a*) osmotically active particles are transferred between the tissues and the body fluids when a hypotonic osmotic stress is imposed, and (*b*) volume control is exhibited in low osmotic stresses (hypo- or hypertonic). The nephridia and/or the gut are found to be responsible for such volume control.

Bethe (1934) demonstrated that *Sipunculus* is permeable to salts when immersed in a medium consisting of one-third isotonic sucrose solution and two-thirds sea water. Adolph (1936) was unable to show the permeability of *Phascolosoma* to salts, but he produced evidence that this worm is differentially permeable to water, that is, it permits water to pass more rapidly inward than outward. Such evidence, however, was produced with animals in which the gut and nephridia were open, a fact to which Krogh (1939) objected.

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It will be shown in the present study that *Dendrostomum* is permeable to salts, but that salt exchanges occur mostly through the gut openings and/or nephridiopores. The body wall is but slightly permeable to salts in this species and is more permeable for particles moving inward. *Dendrostomum* likewise exhibits differential permeability for water in the same direction as salts. This is a property of the body wall.

Peebles & Fox (1933) demonstrated that *Dendrostomum* can lose up to 43 % of its weight by desiccation, yet recovers when returned to sea water. Similar behaviour was shown for *Phascolosoma* by Adolph (1936). The present investigation confirms the work of Peebles & Fox (1933) in respect to the response to desiccation, but also shows that when the animals are desiccated slowly, they tolerate the treatment, but do not recover their normal weights completely when returned to sea water. Also, electrolytes are not excreted when large volumes of water are lost by evaporation. Blood concentrations increase but not in proportion to water loss, indicating some mechanism for removing osmotically active particles from the body fluids.

MATERIALS AND METHODS

Dendrostomum zostericolum Chamberlain* which can be found in deep, under-rock, sandy situations usually in the proximity of eelgrass (*Phyllospadix*), was collected at Flat Rock Point, California. *Phascolosoma gouldi* (Pourtales) was obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts.

The osmotic concentration of solutions was determined by two methods: (*a*) melting-point determination which demonstrates the total osmotic pressure of the solution, and (*b*) measurement of electrical conductivity which depends only on charged particles.

The melting-point method was an adaptation of that used by Jones (1941). The method of Jones compares the melting-points of unknown solutions with the melting-points of a series of sodium chloride standard solutions. This necessitates the selection of the proper range of standards, and thus often involves several runs on one group of unknowns in order to achieve desirable accuracy. This method is advantageous because it permits determinations on small quantities of fluid (less than 0·01 ml.); it also eliminates difficulties arising from coagulation and supercooling, and removes the necessity for a thermometer.

In the present investigation the method was modified by using only three sodium chloride standards, usually of melting-points $-3{\cdot}00$, $-2{\cdot}00$ and $-1{\cdot}00°$ C. Equal volumes of the respective solutions were placed separately into thin-walled capillary tubes of equal lengths. These solutions were quick frozen on dry ice and immersed in a brine bath, the temperature of which was about $-10°$ C. The brine bath was placed in an insulated chamber where the frozen test solutions could be illuminated and viewed through plastic windows. The brine was gently stirred. Observation of the crystals in the capillary tubes was facilitated by placing a polaroid sheet between the tubes and the source of the illumination, and rotating a second polaroid sheet

* Kindly identified by W. K. Fisher, Hopkins Marine Station, Stanford University.

between the capillary tubes and the point of observation. When the second polaroid sheet is rotated to the proper angle, the crystals of the frozen solutions will glow.

Now the time at which each solution melts was noted. This can be done conveniently by marking a rotating kymograph. The temperature of the brine bath in the insulated chamber will elevate slowly in essentially a linear manner over the short temperature range involved. Thus the time of melting for an unknown solution relative to the times of melting for the three standard solutions will give the melting-points of the respective unknowns, e.g. a solution with a melting-point of $-2{\cdot}50°$ C. melts half-way between the standards of $-3{\cdot}00$ and $-2{\cdot}00°$ C.

This method, therefore, permitted determinations on several unknowns of less than 0·01 ml. volume during one run to an accuracy of 0·02° C. within the range of -1 to $-3°$ C.

Conductivity measurements were expressed in percentage of sea water, 100% being considered as having the freezing-point $-1{\cdot}90°$ C. (equivalent to 3·48% salt).

Dilute sea-water solutions were prepared by mixing normal sea water with distilled water. Concentrated media were prepared by boiling normal sea water, care being taken that no salts were lost or precipitated. Concentrations were estimated volumetrically and determined more precisely by conductivity.

Oxygen consumption was measured by means of the Scholander-Wennesland respirometer as described in Umbreit, Burris & Stauffer (1949). All determinations were made at 16° C., a temperature to which the experimental animals were naturally accustomed. No readings were made until the animals remained in the chamber for at least 1 hr.

RESULTS

I. *The inability of* Dendrostomum *to regulate osmotic pressure of body fluid*

The behaviour of *Dendrostomum* under heterosmotic conditions was studied by immersing the animal in sea water of different concentrations, and measuring the uptake and loss of water as a function of time by weight changes, then returning it to normal sea water after equilibrium was reached and measuring the recovery rate to normal or a new equilibrium weight.

Animals were dried uniformly in gauze before each weighing, care being taken to exert little pressure on the body wall. Thus specimens could be weighed consistently to within 0·01 g. Since these forms ingest quantities of sand and detritus, freshly collected animals were allowed to stand in clear sea water for several days before use to permit evacuation of the gut.

Two main groups of animals were used: (*a*) those with gut and nephridia undisturbed, and (*b*) those with gut and nephridia ligated. Since mouth, anus and nephridiopores are located at the anterior end of the animal, a single ligature around the body, posterior to the anus, isolates these openings from the rest of the body. This was accomplished usually by slipping a rubber balloon over the anterior end and fastening with a rubber band.

Results showed that animals immersed in dilute or concentrated sea water, varying 25 % from normal, gain and lose weight respectively at a gradual rate until equilibrium is reached approximately in 1 day (Fig. 1). The time required increases with the size of the animal and the extent of osmotic stress.

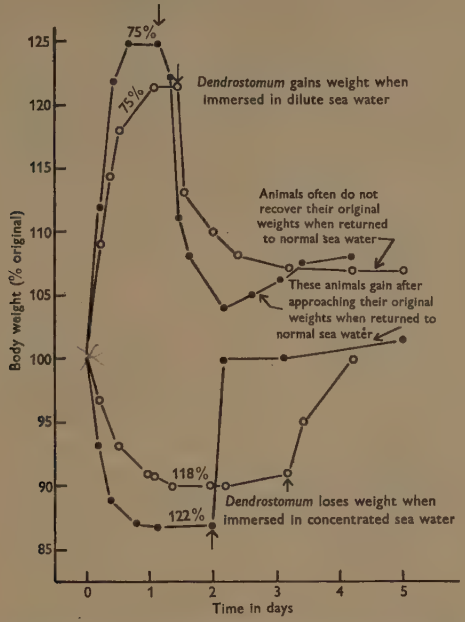

Fig. 1. Responses of *Dendrostomum* immersed in dilute or concentrated sea water as illustrated by four typical cases. The concentration of the medium is written over each curve. Arrows, unless otherwise specified, indicate the point at which the animal was returned to normal sea water. ●, animals with free gut and nephridia; ○, animals with ligated gut and nephridia.

It was then established by melting-point determination that at equilibrium, i.e. when the weight no longer changes significantly, the body fluids of the worm are isotonic to the external medium. This was demonstrated on three specimens, one with gut and nephridia tied off, in each of the following situations (total of twenty-one animals): (1) 90 % sea water, (2) 75 % sea water, (3) 50 % sea water, (4) 125 % sea water, (5) 110 % sea water, (6) recovery in normal sea water from 75 % sea water, and (7) recovery in normal sea water from 125 % sea water. *Dendrostomum*, therefore, demonstrated no ability to maintain an osmotic gradient in heterosmotic situations at least as a steady state condition.

II. *Differential permeability of the body wall to water*

When specimens of *Dendrostomum* were placed in dilute sea water they took up water more rapidly than they lost it when returned to normal sea water for recovery.

Conversely, in concentrated sea water, water was lost more slowly than gained when returned to normal sea water. This held true for ligated specimens as well as for animals with free nephridia and gut (Fig. 1).

This was shown by subjecting twelve specimens (four with ligated anterior ends) to 75 % sea water and eight specimens (one with ligated anterior end) to 125 % sea water. The weight gains and losses respectively were observed during the first 4 hr. of immersion. The period of 4 hr. was arbitrarily chosen because in all cases observed it was less than half the time required to reach a steady state, but still permitted sufficient weight changes to be measured accurately.

When the animals had attained their maximum weight changes, they were returned to normal sea water and, again, the respective weight losses and gains were observed during the first 4 hr. of recovery. The influx of water was thus shown to average 158 % of the outflux of water. This difference was shown by '*t*' evaluation to be valid with the probability less than 0·1 %. Ligation of gut and nephridia had no detectable effect.

These data agree with the observations of Adolph (1936) on *Phascolosoma*, and appear to agree with his suggestion that the body wall of this animal has greater permeability inward than outward for water. Moreover, the objection of Krogh (1939) that Adolph did not exclude the gut and nephridia in his experiments cannot hold in the case of *Dendrostomum*, since ligation did not alter the phenomenon. However, it cannot be said from the evidence presented so far that the body wall *per se* of this sipunculid demonstrates differential permeability because: (1) colloidal and larger ions or other dissolved particles in the body fluids may interfere with the outward passage of water, and (2) irreversible release of bound particles into the blood upon dilution could accelerate and increase water influx.

In an attempt to evaluate these possibilities, water influx in eviscerated animals was measured. Large specimens were bisected near their anterior ends and their viscera, including the adductor muscles, were removed. The bag-like remaining body wall was rinsed and filled with sea water and the open end tied off. When these bags were subjected to osmotic stress, apparent differential permeability to water was demonstrated as it was with intact animals. Since only sea water was inside the bags, there was no sufficient source of larger particles which could hinder outward passage of water. Therefore, the first objection mentioned can be removed.

Eviscerated animals which were turned inside out were filled with sea water, tied off at the anterior end and exposed to osmotic stress. Weight changes in dilute and concentrated sea water and recovery rates when returned to normal sea water demonstrate a definite reversal of differential permeability, relative to the outside of the bag, which again means greater permeability for water from the true outside to inside of the body wall. Fig. 2 summarizes this phenomenon.

If the second objection stated above were valid, that is, the flow of water inward is accelerated by dissociation of bound particles into the internal medium, then the flow inward might be greater even with the reversed bags. The epidermis possibly could be impermeable to released salts whose source in the case of eviscerated

animals would have to be the thick muscle layers of the body wall, but then in such case there would be equal permeability in both directions and no reversal.

With these objections removed, it can be said that the mechanism causing greater permeability to water inward lies somewhere in the epidermis or muscle layers of the body wall. Permeability studies of epidermis separated from the muscular body wall and vice versa were attempted, but sea-water filled bags made of these two structures lost weight regardless of the medium in which they were placed. This is interpreted to mean that sizeable holes were torn in both parts when one was separated from the other, permitting large quantities of fluid to escape into the medium. The body wall of *Dendrostomum* is then more permeable to water from the outside inward, but whether the integument or muscle layers or both are responsible cannot be said at this time.

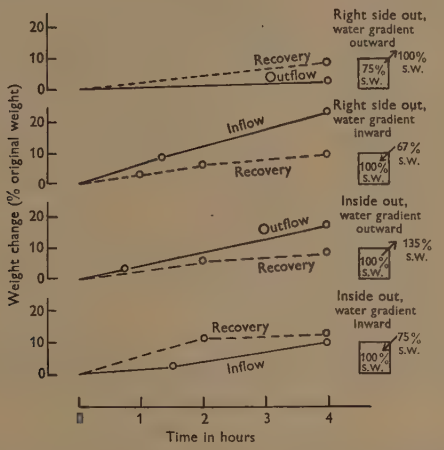

Fig. 2. Bags made from *Dendrostomum* body wall when filled with sea water demonstrate greater permeability for water inward. When bags are turned inside out permeability to water becomes greater outward. Square represents bag. Arrow represents direction of water gradient. 'Recovery' indicates behaviour of specimen when returned after equilibrium to a medium equal in concentration to the original internal medium. S.W. = sea water.

III. *Behaviour of* Dendrostomum *differs from a true osmometer. The failure to recover completely after exposure to osmotic stress*

When sipunculids of this species, with or without ligated gut and nephridia, were immersed in concentrated or dilute sea water varying more than 25 % from normal they reached an equilibrium which they usually held for extended periods. The small loss in weight which usually occurred after equilibrium was not in excess of that lost by normal animals in 100 % sea water. From this fragment of evidence, plus the fact that isotonicity with the abnormal medium was attained, it could be concluded that *Dendrostomum* is semi-permeable and behaves like a true osmometer. This has been stated for *Phascolosoma gouldi* by Adolph (1936) and for *P. vulgare*

and *Sipunculus* by Dekhuyzen (1921). However, it was seen that when *Dendrostomum* is returned to normal sea water for recovery it approaches its original weight but does not attain it. Usually after exposure to dilute situations it remains heavier at equilibrium than it was originally. Sometimes after reaching such an equilibrium it slowly gains more weight (Fig. 1). Individuals subjected to concentrated sea water also often exceed their original weights upon recovery (Fig. 1). Melting-point determinations on blood of animals in this condition showed isotonicity to the medium. It should be borne in mind, also, as mentioned above, that control animals in normal sea water lose weight.

Animals thus exposed to abnormal concentrations usually live indefinitely when returned to sea water, but they exhibit abnormal behaviour; the body becomes soft and irregular in shape, and the usual, quick responses to touch gradually decrease. It does not seem likely, therefore, that these forms in nature could stand such treatment unless normal feeding and activities could prevent or ameliorate such a condition. It was observed, however, that the oxygen consumption of animals immersed in 50 and 150% sea water respectively was reduced to approximately one-half normal. This suggests that the normal activities of the animal are reduced or perhaps permanently injured by the stress, thus causing abnormal behaviour when returned to normal sea water. It may be that the reduced metabolism in osmotic stresses manifests a physical quiescence brought about by the changing environment, thus interrupting normal physiological processes sufficiently to cause damage. In view of the ability of *Dendrostomum* to metabolize at low rates for extended periods without apparent harm (Peebles & Fox, 1933) the latter explanation seems unlikely.

IV. *Total and mobile water content*

Mobile water was determined upon a series of worms by exposing them to media differing by about 25% from the normal, using the relationship

$$\frac{C_1}{C_2} = \frac{V + \Delta W}{V},$$

where C_1 is the concentration of the normal medium, C_2 is the concentration of the artificial medium. ΔW is the change in weight after a steady state has been attained in the artificial medium, V = the volume of mobile water. The values thus obtained for animals with gut and nephridia excluded averaged 70·0% of the total weight of the animal (Table 1) and agree closely, showing no obvious differences between animals immersed in dilute and concentrated sea water or between animals of various sizes. This again suggests semi-permeability. However, figures for mobile water in unligated animals are inconsistent and generally lower than those indicated here. The reason for this discrepancy is discussed below.

Total water content of the above sipunculids was determined by measuring the weight loss caused by drying to constant weight in an oven at 100° C. The average water content was 83·6% body weight, a figure which agrees favourably with Peebles & Fox (1933). The quantity of water in this species also seems relatively constant and does not obviously vary with the size of the individuals measured.

Peebles & Fox (1933), however, found in measuring large numbers of *Dendrostomum* that smaller specimens contain a higher percentage of water. However, animals used by these workers were considerably smaller than those used in the present investigation, and water content may vary more with size in small animals.

Table 1. *Total and mobile water in* Dendrostomum

Specimen no.	Medium % sea water	Original live wt. (g.)	Total water % body wt.	Mobile water % body wt.	Ratio: mobile water / total water
1	125	7·67	82·5	70·8	0·858
2	125	4·56	82·0	65·6	0·800
3	125	5·35	82·0	70·6	0·862
4	125	4·73	84·2	74·3	0·883
5	125	11·95	82·8	69·3	0·836
6	125	15·22	83·4	67·0	0·802
7	125	4·05	84·5	70·4	0·833
8	125	16·25	83·3	70·1	0·838
9	125	17·65	85·4	77·0	0·920
Mean	—	—	83·5	70·5	0·846
10	75	13·92	83·5	69·4	0·830
11	75	9·27	83·7	69·4	0·829
12	75	9·55	83·3	69·3	0·831
13	75	12·61	83·9	68·2	0·812
14	75	13·45	84·8	68·7	0·809
Mean	—	—	83·8	69·0	0·823
Mean of total	—	—	83·6	70·0	0·838

The ratio of mobile water to total water averaged 0·838, but showed some variation. This ratio in the crab *Pachygrapsus* was calculated to be 0·824 (Gross, 1949).

When animals are placed directly into great stresses, calculated values for mobile water often increase greatly, sometimes to magnitudes which could not possibly be true, e.g. over 100 % of the original weight of a specimen in 50 % sea water (Fig. 3). Since such a phenomenon is found in ligated and unligated animals, it seems that the added water could enter only by osmosis which could be accomplished in either of two ways: (1) active uptake of salts which would force more water to enter, or (2) addition of particles from the tissues or by dissociation from a bound state in the blood.

The former alternative does not seem likely, since there is no evidence of such occurrence in lesser stresses where active uptake would be facilitated by high external ion concentrations. The latter possibility was investigated, however.

Melting-point determinations were made on whole sipunculid blood and on blood diluted with distilled water to 75, 50, 25 and 12·5 % concentrations. If release of bound particles from the blood occurs with dilution, then the change in the freezing-point would be lessened from that normally predicted. However, results indicated no addition of particles to the solution when water was added. The possible existence of such a phenomenon in other forms (e.g. decapod crustaceans) where it would be of adaptive value was investigated. Results on *Pachygrapsus*, *Panulirus* and *Cambarus* were all negative.

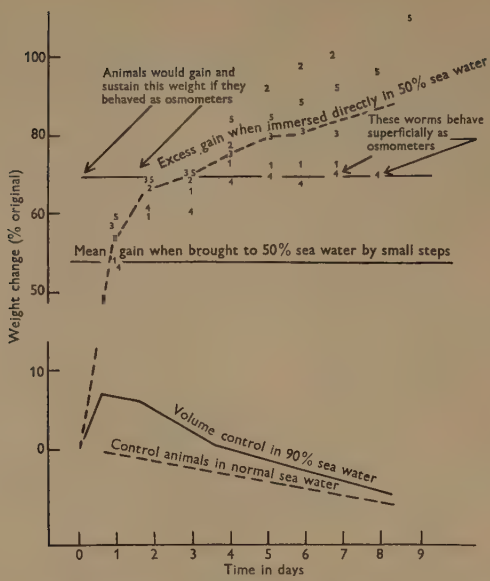

Fig. 3. Departure of *Dendrostomum* from the behaviour of an osmometer as shown by the following phenomena: (1) animals immersed suddenly in 50% sea water gain weight excessively; (2) animals brought to 50% sea water by 10% steps gain less than an osmometer should; (3) animals immersed in 90% sea water demonstrate volume control. Individual specimens are indicated by specific numerals. All specimens had open gut and nephridia.

V. *Volume control and salt permeability*

As mentioned above, when *Dendrostomum* is immersed in sea water varying more than 20% from normal concentration, it reaches a weight equilibrium which it sustains, therefore giving the appearance of an osmometer. Normal animals were therefore immersed in media differing only 10–20% from normal sea water. Such specimens in 90% sea water, as shown in Figs. 3 and 4, swell, reach equilibrium and then lose weight at rates exceeding the loss by normal control animals in 100% sea water. The weight loss of the experimental animals expressed in terms of percentage maximum weight or percentage maximum mobile water is greater than in the control animals, thus suggesting volume control.

Specimens immersed in 120% sea water lose weight as would be expected, but then in some cases show a tendency to return toward normal (Fig. 4). Volume control if it does exist in this stress appears slight, perhaps in part because of normal weight loss (see control).

Volume control, as shown in Fig. 4, could be brought about by excreting only water, excreting salts or both in dilute sea water; in concentrated sea water it could be accomplished by secreting only water inward, secreting salts inward, or both. If an exchange of salts were effected, water might be expected to follow its direction

of movement resulting in isotonicity of the body fluids to the external medium. In such a case water flux would be a passive process and would act according to the physical gradient.

On the other hand, if water were actively exchanged with the medium, an osmotic gradient could possibly be established which in effect would be osmotic regulation. Because of the high permeability to water in *Dendrostomum* it seems improbable that water could be transported effectively by an active process.

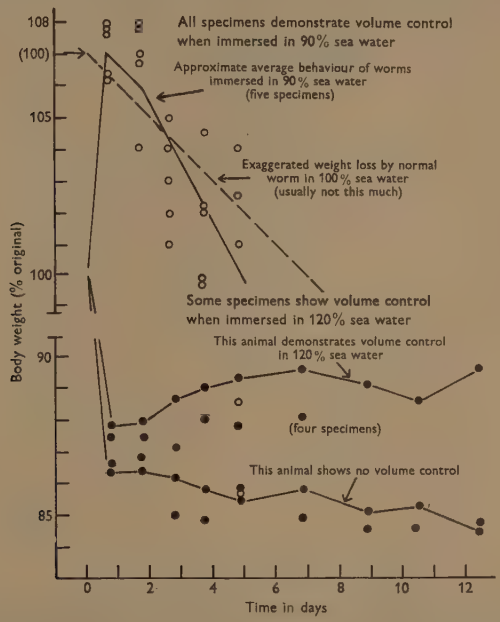

Fig. 4. Volume control exhibited by *Dendrostomum* when immersed in moderate dilutions or concentrations of sea water. All points indicated. ○, animals immersed in 90% sea water; ●, animals immersed in 120% sea water. All open points plotted on upper ordinate.

However, melting-point determinations were made on the body fluids of all animals in Fig. 4 which demonstrated significant volume control, at points where osmotic pressure differences from the medium could be detected precisely. The blood of all such animals was found to be isotonic with the external medium, at point of maximum weight change and at points subsequent to volume control, thus indicating salt permeability of the animal, and also a method of excluding salt without benefit of a gradient.

Adolph (1936) failed to show such behaviour in *Phascolosoma*. However, he did not use small stresses, i.e. less than 20% departure from sea water. Two groups of his species were immersed in 75 and 90% sea water respectively. Results are shown in Fig. 5 and show a behaviour resembling volume control in both media, i.e.

after swelling, a decrease in weight occurs exceeding the weight loss of nor animals kept in 100% sea water. As with *Dendrostomum* the experimental anim. demonstrated a greater weight loss than the controls when such a loss was expresse in percentage of maximum weight or in percentage of maximum mobile water. However, these animals were shipped across the continent and may have been exposed to conditions influencing such behaviour.

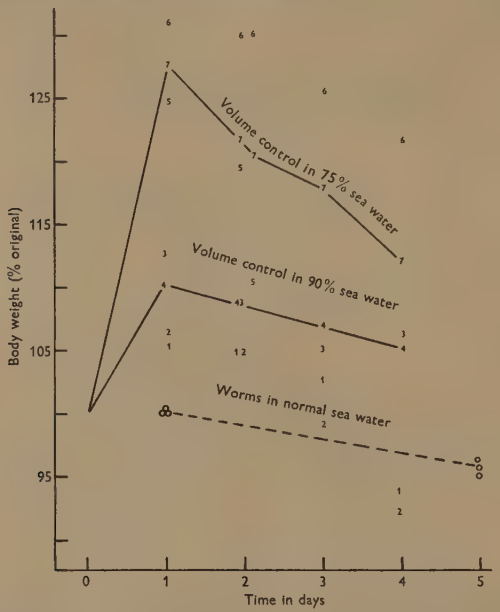

Fig. 5. Volume control as exhibited by *Phascolosoma* in moderately dilute sea water. Numerals represent individual specimens: 1 through 4 = those immersed in 90% sea water; 5 through 7 = those immersed in 75% sea water.

Since the volume control phenomenon suggests a permeability to salts, an experiment was performed to demonstrate the source of such a salt loss. Two groups of *Dendrostomum*, one with gut and nephridia open, the other with these structures ligated, were immersed in increasingly dilute sea water. This was accomplished by exposing them first to 90% sea water until equilibrium was attained, then graduating the animals to 80% sea water until equilibrium was attained, and so forth by steps until finally they were subjected to dilutions as high as 50% sea water.

Mobile water values were calculated for each animal in each dilution from the equilibrium weight it had reached.

These values are presented in Fig. 6 which shows that the calculated mobile water does not change much for animals when gut and nephridia have been excluded. There is a slight decrease down to the 70% sea-water medium, but then an

of crease in 60 % sea water occurs. The decrease in calculated mobile water suggests In slight permeability of the body wall to salts, but not nearly sufficient to permit volume control as shown above. In animals with free gut and nephridia, however, the mobile water values decrease greatly with the increased stress down to 60 % sea water. Then in 50 % sea water the values increase slightly. Fig. 7 shows calculated salt losses necessary to cause the observed differences.

It can be said then that the body wall posterior to the anus where ligation was made is slightly permeable to salt in *Dendrostomum* and that the gut, nephridia or area of the body wall anterior to the anus is permeable to salts or furnishes the mechanism of volume control, namely salt transfer.

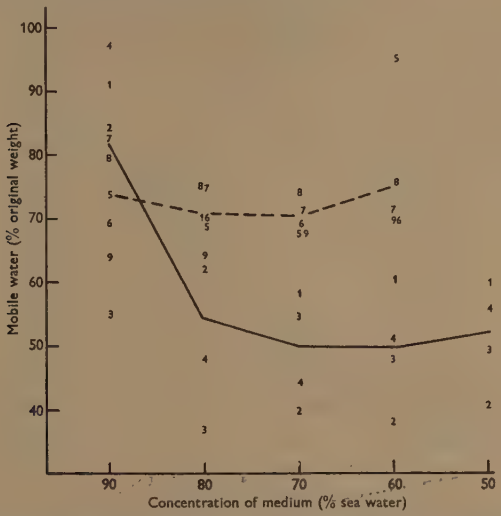

Fig. 6. Calculated mobile water in *Dendrostomum* as function of increasing stress. Animals were allowed to reach steady weights in each successive medium, and mobile water was calculated from such weights in the respective media. All points are indicated; individuals are represented by specific numerals. 1 through 4 = animals with gut and nephridia open; 5 through 9 = animals with gut and nephridia ligated. Broken line = mean of ligated worms; solid line = mean of unligated worms.

In a confirmatory experiment three groups of animals were immersed in a medium (Bethe, 1934) consisting of one-third isotonic sucrose solution and two-thirds normal sea water: (*a*) animals with open gut and nephridia, (*b*) animals with gut and nephridia ligated and covered by rubber balloon, and (*c*) animals ligated anterior to the anus, thus leaving the anus and the nephridia open, but excluding the mouth and area anterior to the anus.

If an immersed animal is permeable to salts, it will tend to lose them to the medium in an attempt to reach equilibrium. This can never be attained, however, if the animal is impermeable to sucrose, because any loss of salt will increase the internal water concentration which, too, will tend to equilibrate to the external

medium. Since the permeability to water is many times the permeability to salts, the rate of such salt loss will govern the loss of weight by the animal.

Fig. 8 shows that the body wall is relatively impermeable to salts, although there must be some loss, thus confirming previous experiments (Fig. 6). When the gut and nephridia were left entirely open, or when only the mouth was occluded, great

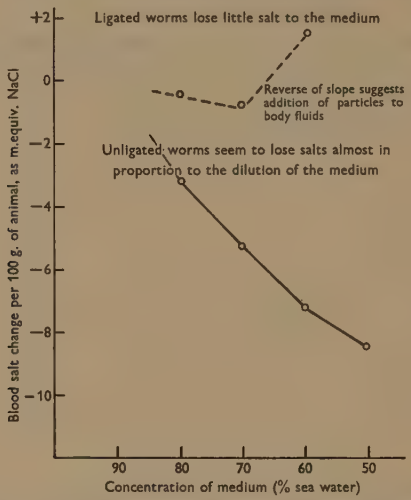

Fig. 7. Salt exchange between *Dendrostomum* and medium required to account for deviation from the behaviour of an osmometer for each increment of stress. Points calculated from mean values using the equation

$$S = KM\left(1 - \frac{M + \Delta W_o}{M + \Delta W_e}\right),$$

where S is the salt loss to the external medium expressed as m.equiv. of NaCl necessary to account for different values for mobile water; K is the NaCl concentration in m.equiv./l. equivalent to the osmotic pressure of normal sea water; M is the volume of mobile water per 100 g. of animal as calculated from the weight gain in 90% sea water; ΔW_o is the observed weight gain (over original) per 100 g. of animal in a given dilution of sea water; ΔW_e is the expected weight gain (over original) per 100 g. of animal in a given dilution of sea water, based on the assumption that the animal behaves as an osmometer. Solid line = unligated worms, broken line = ligated worms.

volume losses were observed which was not the case in fully ligated forms. Animals ligated anterior to the anus seem to lose the most, but this may be attributed to the slightly smaller size of the animals in this group. Members of the other two groups were of comparable size.

It seems then that either the gut and/or the nephridia are responsible for salt losses and, therefore, volume control. In an attempt to determine the role of the nephridia in volume control, a group of sipunculids with plugged nephridiopores was exposed to 90% sea water and observed for weight changes. In not one case out of five was volume control observed. The validity of these results is to be

questioned, however, since the insertion of glass bulbs into the nephridiopores probably caused some injury. Abnormal increases in weight in the 90% sea water attest to this. It should be remembered that when gut and nephridia are both occluded, no such abnormal weight increases occur in moderate stresses. This, however, may indicate a normal influx of water through the gut which is excreted at least in part by the nephridia. The ducts leading to the nephridiopores, however, serve also to transport the genital products to the outside. It would seem unlikely that much water could pass through the nephridial openings when the ducts were blocked in this manner. Koller (1939) demonstrated that removal of the nephridia

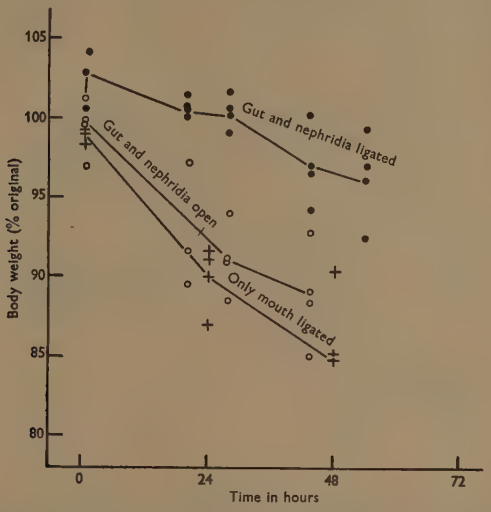

Fig. 8. Salt permeability of *Dendrostomum* as shown by weight loss of animals immersed in a medium consisting of one-third isotonic sucrose solution plus two-thirds sea water. ○, animals with gut and nephridia open; ●, animals with gut and nephridia ligated; +, animals with only the mouth ligated. Solid lines represent approximately average cases. All points are shown.

from *Physcosoma japonicum* prevents volume control in dilute sea water; he concluded from these findings that the nephridia are directly responsible for volume control. However, the same objections discussed above against plugging the nephridiopores can be raised against his experiments. Adolph (1936) stated that the normal turnover of water in *Phascolosoma* is apparently zero, since no evidence of water excretion could be found. This is not believed to be the case for *Dendrostomum*, for it has been observed by the author on several occasions that an animal of this species freshly removed from its burrow in the field will eject a steady jet of clear fluid from its nephridiopores.

Another type of volume control was observed. When animals were immersed in the one-third isotonic sucrose—two-thirds sea water medium, they lost weight

as shown in Figs. 8 and 9. Conversely, normal animals injected with enough isotonic sucrose solution to approximate one-third of their body fluids gained weight in normal sea water. After an appreciable weight change the former animals were returned to sea water and the latter to the sucrose–sea-water solution. At this point the blood of the worms which had been in the sucrose–sea-water medium should have retained its original salt concentration per unit volume. On the other hand,

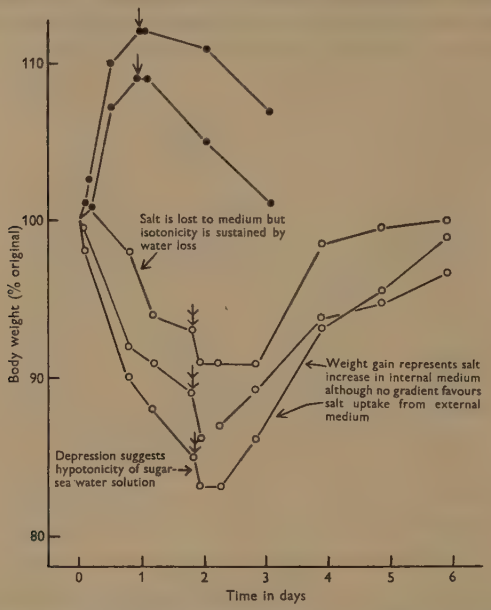

Fig. 9. Volume regulation in *Dendrostomum* independent of total salt gradient between internal and external medium. Worm returns toward normal volume after being experimentally forced away. ●, worms injected with sucrose solutions and immersed in 100% sea water; ○, normal worms immersed in one-third isotonic sucrose two-thirds sea-water solution; ↓, point where injected worms placed in sucrose–sea-water solution; ⇓, point where normal worms returned to 100% sea water.

the injected animals should gain in salts per unit volume since the sucrose becomes dilute with water influx and will cause a smaller part of the body fluid osmotic pressure. Figs. 8 and 9 show that in the new media both groups tend to regain their original weights. This is to be expected for the injected forms since an ionic gradient was established, but the specimens returned to sea water from the sugar–sea-water mixture should have no gradient. Eviscerated sipunculids filled with sea water show this same phenomenon. It seems possible that a mechanism capable of removing salts from the medium lies in the body wall.

It was established above that the body wall of *Dendrostomum* is more permeable to water passing in than passing out. Since it can be shown that the body wall is

somewhat permeable to salts, an experiment was conducted to show whether or not the differential permeability phenomenon also holds for salts.

Sipunculids freshly removed from normal sea water were eviscerated, rinsed with sea water and filled with either (*a*) normal sea water, or (*b*) one-third isotonic sucrose solution plus two-thirds sea water. These were tied off securely by means of a rubber band and immersed as follows: those containing normal sea water were placed in a large volume of one-third isotonic sucrose and two-thirds sea water; those containing the sucrose–sea-water solution were placed in a large volume of normal sea water. The weight changes were observed in these specimens for about 24 hr., after which time they were emptied of their contents, filled with the alternate solution, e.g. the sucrose–sea-water solution, and returned to the opposite external medium. Again, the weight changes were observed for about 24 hr.

When the external medium is normal sea water, the bag should swell, but when sea water is the internal medium the bag should shrink, since in both cases the ions tend to approach an equilibrium between the interior and exterior. Again, because water is far more penetrating to sipunculid body wall than salts, the rates of weight change will depend on the velocity with which salts can pass in or out of the bag. Observation of the rate of weight change of an individual specimen, when subjected to both conditions described above, will demonstrate in which direction the salts are capable of penetrating more rapidly. As a check against traumatic or fatigue effects experimental specimens were divided as to the type of situation they would first endure, part with sea water inside, and part with sucrose–sea-water solution inside. Fig. 10 demonstrates an asymmetry in behaviour of specimens in the two situations. When filled with the sucrose–sea-water solution, all bags swelled when placed in sea water. However, when filled with sea water and immersed in sucrose–sea-water solution there was not a corresponding loss of weight, rather there was an increase at first, then a levelling off or depression. The increased weight in this situation might be accounted for by a slight hypotonicity of the sucrose–sea-water solution to normal sea water. Assuming such hypotonicity, the body wall of *Dendrostomum* appears to show differential permeability to salts as well as water, both passing inward more rapidly than outward.

VI. *The prevention of volume control in large osmotic stresses*

There are four main lines of evidence suggesting that *Dendrostomum* releases osmotically active particles from its tissues into the body fluids: (1) It was shown that when *Dendrostomum* is returned to normal sea water from an osmotic stress, it tends to approach its original weight, but often comes to equilibrium above that weight, then occasionally increases. (2) Volume control is usually not executed by animals subjected to stresses varying more than 20% from normal sea water. (3) Worms immersed suddenly into 50% sea water gain more water than is possible for an osmometer, while those immersed in sea water gradually diluted to 50%, gain smaller volumes of water, showing a loss of salt, and some volume control. (4) Animals graduated by steps to increasingly dilute sea water demonstrate decreased calculated values for mobile water in each successive step, but if graduated

to extreme dilutions, show an increase in this value. This increase appears in 60% sea water for animals with ligated gut and nephridia, but at 50% sea water for unligated animals and is more pronounced for ligated animals. This phenomenon can be interpreted in terms of salt exchange between animal and medium. Fig. 7 illustrates that such a salt loss in normal animals is almost linear. There is a slight decrease in the slope between 50 and 60% sea water, but the sign is the same. Ligated worms, on the other hand, show a small loss, equivalent to only about 1 m.equiv. NaCl/% in a medium of 70% sea water, but then there is an increase when in 60% sea water amounting to more than 2·5 m.equiv. NaCl/%. The source of this salt would have to be the animal itself, unless it were actively removing salts from the dilute medium.

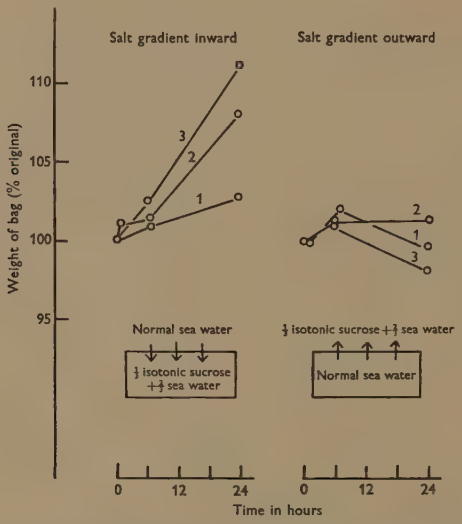

Fig. 10. Differential permeability of *Dendrostomum* body wall to salts as shown by weight changes in bags made from body wall. Numerals represent behaviour of individual specimens and apply to the same specimens in both situations. Rectangles represent bags with their respective internal and external media. Arrows represent the direction of the salt gradient.

Now it appears that if release of osmotically active particles from the tissues of the worm to the body fluids does occur, it is not a direct function of the concentration of the body fluids. It should be kept in mind that animals once exposed to concentrated sea water show excessive gains in weight when returned to normal sea water. Rather it might be that the suddenness with which the body fluids are forced away from present concentrations influences the release of salts from the tissues to the blood. In Fig. 7 ligated animals with only the slightly permeable body wall as a means of releasing salts to the medium show no signs of tissue release of particles until 60% sea water is reached, and then it is much less in magnitude than

in the ligated worms. It might be that efficient release of salts in moderate changes of the medium prevents sudden osmotic pressure changes in the blood so that release of particles from the tissues does not occur until the medium of 50% sea water is reached. Even in ligated worms which have no adequate route of escape for salts to the medium, the gradual influx of water does not seem to impose sufficient stress to cause release of tissue particles into the blood until the animal is stepped from 70 to 60% sea water. It might be that ligated animals demonstrate such releases in lower stresses because no efficient channel for the escape of salts to the outer medium is available.

It is proposed, therefore, that the behaviour of *Dendrostomum* immersed in a dilute medium is influenced by two opposing processes: (1) loss of salt to the medium (via nephridia) tending to reduce volume, and (2) release of particles from the tissues to the blood, tending to increase volume. The latter does not depend on the concentration of the external medium *per se*, but on the suddenness of its imposition or on the actual osmotic stress between tissues and blood. Fig. 3 illustrates how the two opposing processes could work. Here it is demonstrated that three out of five sipunculids immersed directly into 50% sea water gain significantly more than they should if they behaved as osmometers. Yet all animals brought to 50% in steps gained less than an osmometer should. Still in the low stress of 90% sea water all cases show volume control. It is probable, then, that the mechanism for this type of loss of salt to the external medium functions when the animal is brought to 50% sea water by gradual steps. Unless there is occlusion of the salt-excreting channels, it probably also occurs in the specimens placed directly into the 50% sea water, but does not operate fast enough to keep up with the tissue released particles entering the blood, therefore resulting in excessive water uptake.

It is possible, however, for these two processes to function at the same rate, i.e. salts could be excreted as rapidly as particles are released from the tissues into the blood. In such case the animal would appear to operate as an osmometer while actually being more complex. As shown in Fig. 3, two specimens gain as they should if they behaved as osmometers. However, since all animals immersed in 90% sea water demonstrate volume control and, since the behaviour of all specimens brought to a medium of 50% sea water by steps suggests a volume control mechanism, it seems probable that those animals immersed directly into 50% sea water are likewise undergoing processes necessary to reduce their volumes. The excessive weight gains of such animals indicate that particles are being added to the internal medium in excess of the excretion of salts necessary to reduce body volume and therefore giving the appearance in some cases of an osmometer-like behaviour.

Koller (1939) called no attention to the phenomenon, but his data show that *Physcosoma japonicum* gains excessive weight (14%) in 91% sea water which suggests that osmotically active particles are added to the body fluids when an osmotic stress is imposed. *Physcosoma* also shows volume control in 91% sea water, suggesting that the two above-mentioned opposing processes also operate in this species. It is interesting to note that excessive weight gains do not occur when *Physcosoma* is immersed in more dilute sea water (80 to 50%); this is just the

opposite from *Dendrostomum* which shows no excessive weight gains in 90% sea water, but does so in 50% sea water.

Mechanism of tolerance to desiccation

In order to study the effects of desiccation on *Dendrostomum*, animals which were freshly removed from normal sea water were dried and placed where an air current passed over them to speed evaporation. The air temperature was kept the same as the water from which they were removed (16° C.). When worms thus exposed had lost considerable weight they were returned to normal sea water and their recovery observed. It was found that animals which had lost about 25% of their weight in the above manner almost always fully recovered within one day when returned to sea water, that is, their rates of recovery were similar to those of animals forced away from normal by immersion in 125% sea water. One animal returned to its original weight after thus losing 36%. This confirms the work of Peebles & Fox (1933), who reported that specimens of *Dendrostomum* could return to normal in sea water after losing 43% of their weight by evaporation.

However, such full recovery does not occur if sipunculids are desiccated slowly for longer periods to comparable weight losses. Animals treated in such a manner live for prolonged periods when returned to sea water, even gain weight slowly but at slower rates than animals forced away from normal in concentrated sea water. It is probable that slow desiccation merely imposes a stress on the membranes for a damaging period, while rapid desiccation affects the membranes for brief and tolerable periods.

Assuming 70% of the body weight is mobile water, and assuming no loss of osmotically active particles from the body fluids, the internal medium of an animal which loses 25% of its weight by evaporation will attain a concentration equivalent to 155% sea water; the blood concentration of an animal thus losing 36% of its weight will be equivalent to 205% sea water, and the body fluids of an animal losing 43% of its weight will reach an osmotic pressure equivalent to 259% sea water. Since it seems unlikely that the latter stresses could be endured without harm even for brief periods, the possibility that osmotically active particles are removed from the blood during desiccation was investigated.

Worms were removed from normal sea water and were desiccated until they had lost considerable weight. Melting-point determinations were then made on the blood (using only specimens showing signs of life). Results are presented in Table 2. It can be seen that the observed blood concentration was always less than the expected value, based on the assumption that 70% of the original weight is mobile water. It might be argued that the value for mobile water is incorrect, but as shown in Table 2, mobile water calculated for the observed blood concentration exceeds the extreme value for total water in four out of five cases (Table 1). It can be seen also in Table 1 that approximately 15% of the total water is osmotically inactive. It is possible, then, that part of the discrepancy between observed and calculated blood concentrations in desiccated animals is caused by a mobilization of fixed water. It is apparent, however, that some particles are removed from the blood.

This could be accomplished either by (*a*) excreting them from the body, or (*b*) fixing them within the body and therefore rendering them osmotically inactive.

In order to test these alternatives, five animals were removed from normal sea water and rinsed rapidly in distilled water to remove superficial salt. These were then blotted and placed in open, clean containers where they were desiccated to less than 70% of their original weights. Each was then washed in its container with a volume of distilled water approximately equal to the original calculated mobile water. The washings were collected and the electrical resistance of the solutions was measured. In no case did such resistance measurements indicate the presence of electrolytes equivalent to more than 1% sea water. If particles removed from the

Table 2. *Effect of desiccation on the concentration of the body fluids in* Dendrostomum

Specimen no.	Wt. after desiccation, % original wt.	Expected blood concentration, % sea water*	Observed blood concentration, % sea water	Mobile water calculated from observed blood concentration, % original body wt.
1	73	163	145	87
2	74	159	153	75
3	71	169	140	101
4	71	169	136	109
5	71	169	145	94

* Assuming 70% of original body weight is mobile water.

blood are excreted from the body, they must be non-electrolytes. Animals showing such behaviour which are able to recover their normal weights in sea water after desiccation would either have to regain the lost particles from the medium or release particles from their tissues into the body fluids to permit volume recovery. Such a release of particles was demonstrated above, when animals were exposed to large osmotic stresses in dilute sea water. In view of the difficulties of excreting to the outside in the absence of water it seems that the discrepancy between observed and calculated blood concentrations of desiccated sipunculids can be more easily explained by fixation of particles from the blood within the body of the organism.

DISCUSSION

It has been thought previously that the sipunculids act as osmometers when exposed to osmotic stresses. However, the present investigation has shown that while *Dendrostomum* superficially appears to behave like an osmometer when immersed in dilute and concentrated sea water, it actually is more complex. There seems to be an exchange of salts between animal and medium, and an exchange of osmotically active particles between the body wall and the blood of the worm. Within a certain range of hetero-osmotic conditions these two processes apparently nullify each other, thus manifesting a superficial osmometer-like behaviour in the worm. It is

therefore fallacious to assume that the volume changes alone of an organism or cell exposed to an osmotic stress reveal the nature of the limiting membranes.

The differential permeability of *Dendrostomum* to water may offer some explanation as to why certain animals are better hypo-regulators than hyper-regulators* and vice versa. Since *Dendrostomum* is not a regulator, the adaptive value of such a mechanism does not lie in regulation, although the lower permeability outward could help to prevent loss of water through evaporation were the animal exposed at low tide.

Dendrostomum is an animal which does not commonly encounter osmotic stresses, yet when it does experimentally, it demonstrates a response which suggests the beginnings of osmotic independence, perhaps not in the same manner as manifested by the more familiar organisms, but nevertheless to the same end, homeostasis. Pantin (1952) emphasizes the versatility of Nature and cautions against being led astray by a blind quest for common denominators. The osmotic behaviour of the sipunculids may be a case in point for not all of this phylum are non-regulators. In Sumatra the sipunculid *Physcosoma lurco* dwells in soil which is occasionally inundated both by the sea and by fresh water from the river and is necessarily a hyper-regulator (Harms & Dragendorff, 1933). It may be then that the processes observed in *Dendrostomum* are of the same kind as found in *Physcosoma lurco* which became highly developed and thus permitted this sipunculid its osmo-regulatory ability.

SUMMARY

1. The sipunculid *Dendrostomum zostericolum* demonstrates no ability to regulate osmotically.

2. *Dendrostomum* behaves superficially as an osmometer, but is actually more complex: (*a*) the worm shows volume control in concentrated and dilute sea water; (*b*) it is permeable to salts, mostly through the gut and/or nephridiopores; (*c*) it can release osmotically active particles from its body wall to the blood.

3. The body wall of *Dendrostomum* is highly permeable to water, but only slightly to salts. Permeability for both salts and water is greater inwards than outward.

4. *Dendrostomum* can tolerate a loss of 36% body weight by desiccation and recover when returned to sea water. The mechanism of this tolerance appears to be the removal by fixation in the tissues, of osmotically active particles from the body fluids.

This investigation was done under the direction of Prof. Theodore H. Bullock to whom I am beholden for his close interest and invaluable guidance. Prof. Denis L. Fox suggested the use of *Dendrostomum*, a happy choice for which I owe him my thanks.

* According to terminology used by Jones (1941) hypo-osmotic regulation is the ability to maintain the concentration of the body fluids dilute to the external medium; conversely, hyper-osmotic regulation is the ability to maintain the body fluid concentration hypertonic to the external medium.

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THE CHEMICAL COMPOSITION OF THE BLOOD OF SOME AQUATIC CHORDATES, INCLUDING MEMBERS OF THE TUNICATA, CYCLOSTOMATA AND OSTEICHTHYES

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Included in the Chordata are groups of widely different organization which have certain common morphological features at some stage of their existence, a notochord, a hollow dorsal nerve-cord, and gill-pouches or gill-clefts. The various subphyla differ in most other morphological characters, and in their physiology, especially in methods of respiration, feeding, digestion, excretion, reproduction, and locomotion. A study of the composition of the blood in members of the aquatic Chordata shows that variations exist in the equilibrium between the internal and external media which seem to be conditioned by the level of organization of different groups. Most of the data on these animals, however, is incomplete, and rather unsatisfactory on grounds which are discussed below in each section.

The aim of the present work has been to obtain reasonably complete and accurate analyses of the blood of various members of the less highly organized chordate groups. Comparison of these analyses with that of the external medium will give a measure of any ionic regulation effected by these animals.

MATERIAL AND METHODS

In tunicates blood was withdrawn by means of a syringe from the heart and placed in tubes held in a beaker of crushed ice. It was then immediately centrifuged and the plasma separated for analysis. The specimens of *Phallusia mammillata* had been collected 2 or 3 days previously, and were taken from an aquarium tank at Naples; those of *Salpa maxima* were collected from the waters of the Gulf of Naples, and analysed immediately they were received in the laboratory. Samples of the surrounding sea water were taken simultaneously and the chloride content of this determined.

The hagfishes, *Myxine glutinosa*, were collected in modified lobster creels baited with dead fish from the St Abb's Head ground off the coast of Berwickshire (Cunningham, 1885, p. 17; Fairbairn, 1951). Immediately on capture, blood was obtained from the caudal artery and vein, and the subcutaneous sinus of each specimen; it was allowed to clot and the serum then separated for analysis. Surface samples of sea water were also taken, but the chlorinity of the sea water at the depth of the creels (70 m.) would be a few per cent higher. The chloride equilibrium

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between *Myxine* plasma and the external medium was obtained by keeping a specimen 9 hr. in surface sea water, before collecting samples of the blood and water.

Lampreys belonging to the species *Lampetra fluviatilis* were obtained from the River Severn, trapped during their upward migration from the sea. They were then kept 24 hr. in Glasgow tap water. Blood was collected under liquid paraffin from the caudal artery and vein by means of a fine-pointed pipette, chilled and centrifuged without delay, and the plasma used. The same procedure for collecting blood was used with *Coregonus clupeoides*, the powan, obtained at the University field station at Loch Lomond.

The Roman conger, *Muraena helena*, was kept 3 or 4 days without food in the tanks at Naples, and the blood obtained from the ventral aorta through a cannula which dipped under liquid paraffin in an ice-cold tube. Plasma was separated by centrifugation.

Specimens of *Saccoglossus horsti* belonging to the Hemichordata were collected with the kind assistance of Prof. J. E. G. Raymont of the University of Southampton, but unfortunately it proved impossible to obtain any coelomic fluid for analysis. The collar contains a very viscous fluid which cannot be withdrawn easily by pipette.

Methods of analysis for sodium, potassium, calcium, magnesium, and chloride were those of Robertson & Webb (1939), with suitable modifications for the smaller absolute quantities of these ions available in the blood of fishes. The chief of these was precipitation of magnesium in centrifuge tubes at 80–95° C., the hydroxyquinolate being separated by centrifugation. Webb's (1939*a*) barium iodate method was used for sulphate, with standards of similar chloride and sulphate content to the plasma or serum. Bicarbonate, ammonia and urea were estimated by microdiffusion techniques (Conway, 1947), inorganic phosphate by the method of Sumner (1944), and protein usually gravimetrically (Robinson & Hogden, 1941). Water contents were obtained by evaporating samples on a water-bath, and then drying in an oven at 100–101° C. for 4 hr.

It is emphasized that the data given below were obtained from animals in a thoroughly healthy state, as far as could be judged. Evidence of altered permeability, due apparently to poor condition of the animals, was found in the fresh-water species. For example, powan which had lost many of their scales by being kept in a wire cage in the loch during stormy weather had concentrations of sodium and chloride about 77% of the values found in specimens from the tanks of the field station.

TUNICATA

Two biochemical peculiarities of ascidian blood are the presence of an organic compound of vanadium and a high concentration of sulphuric acid in certain of the corpuscles. Henze (1911, 1912), who discovered these features in *Phallusia mammillata*, concluded in his second paper that the plasma was neutral, and gave an analysis of it compared to sea water, finding the concentrations of ions to be K 109%, Ca 99%, Mg 99%, Cl 104% and SO_4 51%. In *Chelyosoma siboja*, belonging to a family allied to the Ascidiidae, Kobayashi (1935) claims that the

acidity of the plasma is 0·034 N, and he finds considerably more regulation, comparable figures being Na 96%, K 133%, Ca 109%, Mg 76%, Cl 90% and SO_4 175%. Probably the acidity is due to the rupture of some of the acid-containing corpuscles, as Webb (1939*b*) suggests; the relatively low sodium and chloride figures, and high sulphate would be explained by dilution of the plasma with the contents of some of the corpuscles, which, as Kobayashi has found, contain high sulphate and low sodium and chloride concentrations. This interpretation is made more probable by Kobayashi's (1938) data on what seems to be pericardial fluid. He called it 'perivisceral fluid which surrounds the heart', but it does not seem to be comparable with the fluid of that name in *Ciona* which is present in cavities which open to the branchial sac (see, for example, Berrill, 1950). Kobayashi's 'perivisceral fluid' had a pH of 7·2 and differed from his previous analysis especially in the figures for sodium, chloride and sulphate. Expressed as the percentage of each ion in sea water, the regulation amounted to Na 97%, K 128%, Ca 98%, Mg 83%, Cl 99% and SO_4 77%.

Bialaszewicz (1933) has also analysed *Phallusia*, but his figures differ somewhat from those of Henze (1912), especially in calcium and sulphate, these ions being 111 and 99% of those in the sea water of Naples aquarium. A chloride concentration 110% of that in the sea water suggests disequilibrium, or analytical error. His analysis of *Ciona intestinalis* compared with sea water is K 104%, Ca 101%, Mg 87%, Cl 106% and SO_4 73%.

In view of the discrepancies apparent in the published data, a re-examination of the plasma of *Phallusia mammillata* has been made. Carefully centrifuged plasma gave a pH of 6·6. Two complete analyses were made of the plasma from five and from four specimens, and differences from the mean figures in Table 1 did not

Table 1. *Ionic regulation in the Tunicata*

	Concentration in plasma as percentage of concentration in sea water						mg./ml.	
	Na	K	Ca	Mg	Cl	SO_4	Protein	H_2O
Ascidiacea								
Phallusia mammillata	99·4	100·2	93·3	98·8	103·6	52·5	0·3	979
Thaliacea								
Salpa maxima								
Solitary	100·3	112·9	95·9	94·9	102·4	64·9	0·3	982
Chain	100·8	114·3	95·0	95·4	102·5	67·2	0·2	982

exceed 1·3% except in the case of sulphate, where the deviation was 2·6%. The data are essentially in agreement with those of Henze (1912), except that the calcium in the plasma is lower, and no regulation of potassium exists; in addition, they show that sodium is slightly and just significantly lower than the sea-water value. Halving of sulphate brings about an increase in chloride to maintain ionic balance; the contribution to this of bicarbonate is small, being 1·10 m.equiv., about half the concentration of this ion in the aquarium sea water. Isosmotic equilibrium exists between the body fluid and the surrounding sea, both fluids having an ionic

concentration of 1·347 g.-ions/kg. water; this agrees with the freezing-point determinations of Henze (1911, 1912).

A somewhat similar pattern of regulation is shown by *Salpa maxima*, one of the transparent pelagic tunicates, which exists as asexual solitary forms (oozooids) and sexual chain forms (blastozooids). The pH of the plasma from four solitary individuals, and from the twenty members of a chain form was 7·5 in each case. Similar regulation is shown by the two forms of *Salpa*, all ions except sodium being kept at different values from those of the external medium. Differences from *Phallusia* are the considerable accumulation of potassium, and the more definite reduction of magnesium.

Compared with other invertebrates, the composition of the plasma of these two tunicates resembles that of the mesogloea of *Aurelia* (Robertson, 1949) in the reduction of the divalent ions, calcium, magnesium and sulphate, and in the low protein content.

CYCLOSTOMATA

Maintenance of approximate osmotic equilibrium between the blood and sea water is a feature separating the myxinoid group of the Cyclostomata and the elasmobranchs from the marine teleosts. But this equilibrium is achieved by different methods; in the elasmobranchs urea and trimethylamine oxide constitute about half the total concentration of osmotically active particles (Smith, 1936), whereas in the myxinoids inorganic ions apparently account for most of the concentration. Freezing-point determinations of hagfish blood show it to be about 2% hyperosmotic to sea water (Dekhuysen, 1904; Greene, 1904; Schmidt-Nielsen & Schmidt-Nielsen, 1923; Smith, 1932), but the ratio of chloride in blood serum to chloride in sea water appears to be rather variable. In *Myxine* ratios of 0·70–0·88 are given by the Schmidt-Nielsens, and 0·85–0·93 by Smith (1932) and by Cole (1940); a low figure of 0·62 is given by Borei (1935) who seems to have analysed whole blood. In the Californian genus *Polistotrema* (= *Bdellostoma*) Bond, Cary & Hutchinson (1932) find a ratio of 0·89. Data on the concentration of urea in the blood of *Myxine* are contradictory, Smith (1932) finding only 0·15–0·28 g./l., and Borei much higher amounts, 3·5–3·7 g./l. While Krogh (1939) doubts Borei's figures, Needham (1938) accepts them, and on their basis speculates on the evolutionary history of the marine cyclostomes.

Some of these discrepancies are undoubtedly due to faulty technique, and one is probably apparent only. Unlike the other investigators who analysed the serum of *Myxine*, Borei examined whole blood, and his low chloride figure must reflect the low chloride content of the cells. Fänge (1948) has given the chloride concentration of whole blood and serum of specimens from the same locality as 13·3 and 16·3 g./l. respectively. The lower of the chloride values of the Schmidt-Nielsens may be due to losses of chloride by volatilization, as they ashed blood serum in the presence of sodium carbonate. Regarding the discrepancies over the urea content of the blood, the analyses given below agree well with those of Smith (1932). Borei's (1935) method of estimating the nitrogen in an alcohol-ether extract of blood, and cal-

culating it as urea, assumes that all non-protein and non-lipoid nitrogen in plasma and cells is urea, and is quite unspecific.

The only ionic analysis of *Myxine* serum available is that of Smith given by Cole (1940). As a percentage of the values in sea water, on a volume basis, the ions are Na 96·8%, K 100%, Ca 56·4%, Mg 45·0%, Cl 92·8% and SO_4 19·7%. Calculation from the data would seem to indicate a total ionic concentration nearly 10% below that of sea water (891 as against 987 mg. ions/l.), but the concentrations would be closer if calculated on the basis of water content of the two fluids, which is unfortunately not given.

Table 2. *Composition of the serum of* Myxine glutinosa

	m.equiv./kg. water									mg. ions/ kg. water
	Na	K	Ca	Mg	Cl	SO_4	P*	Cations	Anions	
Myxine serum										
(1)	563	8·7	11·0	37·1	570	13·3	13·5	620	597	1180
(2)	550	9·5	12·2	35·3	565	12·9	11·5	607	589	1161
(3)	562	10·5	14·3	43·9	592	13·8	—	631	606	1201
Mean	558	9·6	12·5	38·8	576	13·3	12·5	619	602	1183
Sea water	506	10·7	22·2	116	592	61·1	—	655	653†	1209†
Myxine serum as % sea water	110	90	56	33	97	22	—	—	—	98

* Present as $H_2PO_4^-$ and HPO_4^{2-} with a mean valency of 1·87 at pH 7·6. Mean value of 12·5 m.equiv. equals 6·7 mg. ions.
† *Minus* HCO_3 of 2 m.equiv. or mg. ions.

In Table 2 are given the data obtained in three separate analyses of the serum of *Myxine*. The first analysis was made on pooled serum from three specimens captured in September, the second and third on single specimens caught in April and September respectively. All three show good agreement. Comparison with sea water has been made on the basis of the chloride equilibrium, serum : sea water, 97·2 : 100, found in a specimen kept in surface sea water for 9 hr. This ratio becomes 91 : 100 if based on a volume rather than a water-content basis, and it then falls within the range given by Smith (1932) and Cole (1940).

Compared with sea water the composition of the serum differs in every ion, values for all ions except sodium being below those of the water. Phosphorus anions HPO_4^{2-} and $H_2PO_4^-$ are as important quantitatively as SO_4^{2-}; assuming a pH of 7·6 (Smith, in Cole, 1940), most are divalent. The deficiency in anions is presumably made up by HCO_3^- (3·7 m.equiv. in Smith's specimens), and protein. Two estimations of the latter lay between 66 and 68 g./l. Total ionic concentration of the serum is on the average 2% below that of the sea water, but certain further calculations suggest that in fact the osmotic pressures exerted by the ions of the two solutions must be nearly the same. To the total ions of the serum (1183) must be added about 4 mg. ions for HCO_3^- and 1 mg. ion for $Pr.^-$, that is, a total of 1188. From freezing-point data it can be calculated that an artificial sea water made up from isosmotic solutions of the salts NaCl, KCl, $CaCl_2$, $MgCl_2$ and $NaSO_4$, with concentrations K 90%, Ca 56%, Mg 33% and SO_4 22% of those of a sea water

with 592 m.equiv. Cl/kg. water, would have other values as follows: Na 110%, Cl 101·7% and a total of 1204 mg. ions. The essential factor in the isosmoticity of solutions of 1211 and 1204 mg. ions is the replacement of some of the less 'active' Na^+ and SO_4^{2-} ions in the first solution by more 'active' Na^+ and Cl^- ions in the second, resulting in the same osmotic effect with fewer total mg. ions. Now, if this artificial sea water were to have a composition similar to the serum, the extra chloride ions of this water compared with the serum, $602-576=26$ mg. ions or m.equiv., would be replaced by 26 m.equiv.* bicarbonate, phosphate and protein ions. These would equal 12 mg. ions ($4\ HCO_3^- + 1$ Pr. $+ 7\ H_2PO_4^-$ and HPO_4^{2-}), a saving of 14 mg. ions, which means that this hypothetical serum of total concentration $1204-14=1190$ mg. ions would be isosmotic with the sea water of 1211 mg. ions (Cl 592 m.equiv.). On the basis of ions alone, the mean value for *Myxine* serum, 1188 mg. ions/kg. water, is therefore within 0·2% of that deduced to be isosmotic with the sea water. A small correction to the *Myxine* figure, to take into account the fact that about 25% of the calcium is bound in a protein-complex, would still give a value only about 0·5% lower than the calculated value for equilibrium.

The osmotic pressure of the serum will be further increased by the content of urea. Values measured by the urease method were 0·255 and 0·122 g. urea/kg. water in analyses (1) and (2) respectively, constituting only 4 and 2 mM. These are maximum figures, as any trace of preformed ammonia is included in the calculation as urea. In these specimens of *Myxine*, therefore, the blood serum has a total particle concentration almost identical with that of the external medium, differing at most by 1%. Inorganic ions account for 99·7% of the osmotic concentration.

Smith's analysis of serum, if calculated on a water-content basis by a division factor of 0·921 (0·921 g. water/ml. is the mean water content of the sera in Table 2), would be exactly 3% below osmotic equilibrium with the surrounding sea water.

It should be pointed out that the comparison of *Myxine* serum with sea water made above (Table 2) is not exactly a true measure of the ionic regulation effected by the animal. In marine animals ionic regulation is best defined as the maintenance of concentrations of ions differing from those of a passive equilibrium with the external medium, and in the case of *Myxine* this equilibrium, with its Donnan effects (see, for example, Robertson, 1949), was not determined owing to lack of material. As far as the comparisons of total concentrations are concerned, serum dialysed against sea water would have a total concentration greater than that of sea water by 0·1–0·2%, on account of protein and the Donnan effect.

No analyses appear to exist of lampreys living in sea water, except a single freezing-point of the blood of *Petromyzon marinus*, Δ 0·586° C. compared with about Δ 2·30° C. for the Mediterranean (Burian, 1910). Data for *P. marinus* from the River Loire in April are Δ 0·54° C., Cl 119 m.equiv. (Fontaine, 1930*a*), but these figures decrease at the end of the reproductive period towards the end of

* In so far as the mean value for bicarbonate, phosphate and protein ions in the blood of *Myxine* is 30 m.equiv., a slight discrepancy exists between the real and the hypothetical serum. This affects the calculation to a negligible extent.

June, apparently because the animals can no longer withstand the osmotic gradient. Previous data on *Lampetra fluviatilis* show freezing-points of Δ 0·46–0·50° C. when these animals are in fresh water (Dekhuysen, 1904; Galloway, 1933), and Cl and Ca figures of 121 and 5·3 m.equiv., respectively, in specimens from the River Severn (Galloway). Both species are peculiar in being unable to withstand transfer back to sea water, even if this is done gradually (Fontaine, 1930*a*; Galloway, 1933); presumably some important change in their capacity for regulation has occurred since they migrated up-river from the sea.

Table 3. *Composition of the plasma of* Lampetra fluviatilis

m.equiv./kg. water											mg./ml.	
Na	K	NH_4	Ca	Mg	Cl	SO_4	HCO_3	P*	Cations	Anions	Protein	H_2O
119·6	3·21	0·38	3·93	4·21	95·9	5·44	6·41	12·8	131·3	120·5	36·0	959

* Present as $H_2PO_4^-$ and HPO_4^{2-} with a mean valency of 1·84, assuming a pH of 7·5. 12·8 m.equiv. equal 6·95 mg. ions P.

Lampetra plasma, from animals obtained in late February, had the composition given in Table 3. Pooled blood from eight specimens was used for analysis of the principal ions, but the determinations of ammonium and urea, of bicarbonate, and of phosphate were made on the blood from individual animals. Sodium constitutes 91 % of the cations, and the values for potassium, calcium and magnesium are very similar. In milliequivalents phosphate is much greater than bicarbonate and sulphate, and constitutes nearly 11 % of the anions investigated. The deficiency of 11 m.equiv. anions is probably made up chiefly by protein, with perhaps traces of other radicals, such as lactate. Total concentration of ions comes to 239 mg. ions/kg. water, a figure which indicates a smaller concentration of salts than that suggested by the freezing-points of previous workers. This is perhaps linked with the low level of salts in Glasgow tap water (from Loch Katrine), the bicarbonate and chloride concentrations being only about 5 and 3 mg./l. respectively. It does not seem to be due to a progressive loss of salts, since the sodium and chloride values in the plasma of two lampreys kept for 3 days in the tap water were within 2 % of the values of these ions in Table 3, which are from animals kept only 1 day in the water.

On two occasions no urea was found in the plasma, but small quantities of ammonium ions were present, 5·4 and 7·7 mg./l., giving a mean figure of 0·38 m.equiv./kg. water. Florkin (1943) claims that in the tench and trout the concentration of ammonium ions *in vivo* is very small, and rises to values of about 4–5 mg./l. in 2 or 3 min. after contact with air. While such contact with air was avoided by withdrawing the blood under liquid paraffin and centrifuging the chilled blood immediately, the plasma was not analysed until 15 min. later, and the values may be slightly high.

OSTEICHTHYES

One of the difficulties encountered in making analyses of the blood of fishes is the ease with which haemoglobin and potassium escape from the cells into the plasma. Thus both Macallum (1910) and Smith (1929*b*) realize that some of the potassium values they give are too high, since the serum was tinged with haemoglobin. Furthermore, most of the older determinations of ammonia and urea in fishes may be questioned on two grounds, non-specificity of the chemical methods (Grollman, 1929), and failure to take into account possible increase in ammonia in collected blood (Florkin, 1943). While the low values of ammonia and urea in the blood of the fresh-water carp are probably correct (Smith, 1929*a*), high figures in the blood and urine of marine teleosts, such as those of Edwards & Condorelli (1928), are attributed by Grollman to non-specific methods, or to the breakdown of compounds like trimethylamine oxide, the volatile trimethylamine being determined with the ammonia in titration methods. This latter error is more likely to occur in urine analyses, since Norris & Benoit (1945) find no appreciable quantities of trimethylamine oxide in the serum of several marine teleosts. Brull & Nizet (1953), however, claim that half the nonprotein nitrogen in the plasma of the angler-fish *Lophius* is in the form of trimethylamine and its oxide.

In an analysis of the plasma of the Roman conger, *Muraena helena*, using specific micro-diffusion methods, figures of 34·3 mg. NH_4/l. and 53·4 mg. urea/l. have been obtained, compared with the previous figures for this species of 59–75 and 894 mg./l. respectively (Edwards & Condorelli). While the level of nitrogenous compounds in body fluids may vary with the state of nutrition, this high urea value of Edwards & Condorelli would seem to be erroneous, as Grollman suggests.

Table 4. *Composition of the plasma of* Muraena helena

	m.equiv./kg. water											mg./ml.	
	Na	K	NH_4	Ca	Mg	Cl	SO_4	HCO_3	P*	Cations	Anions	Protein	H_2O
Apodes													
Muraena	211·8	1·95	2·07	7·73	4·85	188·4	11·35	8·03	9·59	228·4	217·4	80·0	917
Sea water	564	12·0	—	24·8	128·7	659	68·0	2·8	—	730	730	—	987
Plasma as % sea water	38	16	—	31	4	29	17	287	—	31	30	—	—

* Present as $H_2PO_4^-$ and HPO_4^{2-} with a mean valency of 1·84, assuming a pH of 7·5. 9·59 m.equiv. equal 5·21 mg. ions P.

Table 4 gives the concentrations of the principal ions in the plasma of *Muraena*. Sodium constitutes over 92% of the cations, potassium less than 1%, while chloride accounts for about 87% of the anions, which include sulphate, bicarbonate and phosphate in almost equivalent concentration. The anion deficit appears rather small in relation to the high concentration of protein (80·0 g./l.) and suggests a slight underestimation of sodium or overestimation of the other anions. In the plasma the total concentration of ions and molecules is 429·4 mg. ions +0·97 mM. urea, which is equal to 32% of the ionic concentration in the sea water (1349 mg. ions). The ionic gradient between plasma and sea water is least in the case of

sodium, and greatest in the cases of potassium, sulphate and magnesium, especially the latter.

No strictly comparable data are available in the literature, as the concentration of the sea water is not given, and the determination of anions is incomplete. Drilhon's (1943) figures for the cations of *Muraena* are Na 182, K 5·1, Ca 7·4 and Mg 1·2 m. equiv./l. plasma, while Edwards & Condorelli (1928) give Na 253, Ca 7·8, Cl 177 and P 5·7 m.equiv./l. serum. Smith (1929*b*) gives three almost complete analyses of the serum of the angler-fish, *Lophius piscatorius*, which agree generally with the data in Table 4, except that potassium is higher (up to 9 m.equiv.) and sulphate lower; the agreement between cations and anions, however, is only fair, one specimen having an anion deficit of 6 m.equiv. which could be attributed to protein, a second 31 m.equiv., and the third a cation deficit of 4 m.equiv., which would be increased still further by the protein. The total ion concentration ranges between 400 and 438 mg. ions/l., with a mean of 419.

Table 5. *Composition of the plasma of* Coregonus clupeoides

	m.equiv./kg. water											mg./ml.	
	Na	K	NH_4	Ca	Mg	Cl	SO_4	HCO_3	P*	Cations	Anions	Protein	H_2O
Isospondyli *Coregonus*	140·9	3·81	0·33	5·34	3·38	116·8	4·58	10·58	8·67	153·8	140·6	41·9	962

* Present as $H_2PO_4^-$ and HPO_4^{2-} with a mean valency of 1·84 at pH 7·5. 8·67 m.equiv. equal 4·71 mg. ions P.

Fresh-water teleosts are unable to maintain such high concentrations of salts as their marine relatives. *Coregonus clupeoides* (Table 5) has a total concentration of 284 mg. ions/kg. water, compared with 429 in *Muraena*. All ions except potassium are lower in *Coregonus*, but the chief reductions are in sodium and chloride. The figures for the principal ions were obtained by analysis of the pooled plasma of two female specimens, but other specimens were used in the determination of bicarbonate, pH, ammonium and urea. Values of urea were 45 and 16 mg./l. in fishes kept without food for 1 and 4 days respectively.

Püschel's (1928) analysis of the tench, *Tinca vulgaris*, agrees with that of *Coregonus* in the concentration of its cations, but the chloride and phosphate anions given amount to only 91 m.equiv. against 149 m.equiv. base. Even allowing another 25 m.equiv. anions for bicarbonate, sulphate and protein, a large deficit remains, suggesting serious errors, probably in the estimation of chloride, and perhaps sodium. The fuller analyses of Smith (1929*b*) on the holosteans *Amia* and *Lepidosteus* compare well with the analysis of *Coregonus*, except that they have about double the concentration of calcium, and only about a quarter of the magnesium.

DISCUSSION

As a group the tunicates maintain their body fluids in osmotic equilibrium with the surrounding medium, and show a certain amount of ionic regulation, particularly in their low levels of sulphate ions. This regulation is surprising in animals lacking excretory tubules, but is paralleled in the mesogloea of *Aurelia* (Robertson,

1949) where similar large reductions in sulphate and small reductions in calcium and magnesium are found. What connexion exists between the high concentration of sulphuric acid (9% H_2SO_4) in the vanadocytes of *Phallusia mammillata* (Webb, 1939*b*) and the low sulphate content of the plasma is not clear. Maintenance of low sulphate in the plasma against continuous inward diffusion of this ion from sea water is possible if the corpuscles accumulate sulphate and are then eliminated from the body, but such an excretion has not been recorded. *Salpa*, however, has a low plasma sulphate and is without acid-containing corpuscles, facts which suggest that ionic regulation in this thaliacean is effected by the cells of the surfaces in contact with the external medium. Such a hypothesis is also necessary in ascidians to account for the reduced calcium in the plasma of *Phallusia*, and sulphate may be regulated in the same way.

Two further features of ascidian blood are worth noting, the low carbon dioxide capacity, and the very small amount of protein, the latter a feature shared also by the pelagic salps. The production of metabolic carbon dioxide in marine animals normally leads to a gradient in the concentrations of bicarbonate from the internal to the external medium. In *Phallusia* and *Ciona* plasma the total carbon dioxide content is only about half that of sea water, but normal respiratory output must take place, since Florkin (1934) finds an increase in the carbon dioxide content of sea water when a specimen of *Ciona* is kept under paraffin oil. This apparent paradox is not to be explained by a presumed utilization of this substance in the synthesis of the cellulose tunic causing low values in the plasma (Henze, 1932), or by postulating active excretion against a concentration gradient. The two features of cardinal importance here are the much lower pH of the plasma, and the varying amounts of dissolved carbon dioxide and of bicarbonate ions in the two media.

Calculations have been made of the three fractions of carbon dioxide, using the apparent dissociation constants of carbonic acid as established for sea water by Buch, Harvey, Wattenberg & Gripenberg (1932) and Buch (1933), and the pH and total carbon dioxide values found in the Naples specimens of *Phallusia*.

Phallusia pH 6·6, 14° C.	mM.	Sea water (23·0 mg. Cl/ml.) pH 8·1, 14° C.	mM.
$[H_2CO_3]$	0·312	$[H_2CO_3]$	0·022
$[HCO_3^-]$	1·104	$[HCO_3^-]$	2·465
$[CO_3^{2-}]$	0·004	$[CO_3^{2-}]$	0·283
Total $[CO_2]$	1·420	Total $[CO_2]$	2·770

The carbonic acid, or dissolved CO_2 fraction, shows a normal gradient, whereas the HCO_3^- ion is much less concentrated in the plasma, that is, the alkali reserve of the plasma is much lower than that of sea water.

A protein content below 1 g./l. is typical of the sedentary or slow-moving invertebrates, such as echinoderms and lamellibranchs, which lack respiratory proteins. Tunicates fall within this group of relatively lowly organized invertebrates in both this feature and in their ionic regulation. Perhaps the reason why this marine subphylum has no fresh-water members lies in the difficulty of controlling osmotic

intake of water and loss of salts in animals with such large pharyngeal and atrial surfaces in contact with the external medium, especially since an excretory tubule type of organ, which could be used for water excretion, is lacking.

The division of the cyclostomes on morphological grounds into two orders, the Myxinoidea and the Petromyzontia, is paralleled by the dichotomy in osmotic and ionic regulation shown by members of these groups. Among the marine vertebrates the hagfishes are alone in maintaining in their plasma concentrations of inorganic ions equivalent to those of the surrounding sea water. While apparently permeable to water and salts like the marine invertebrates, only osmotic equilibrium or steady state exists, for each individual ion is regulated, at least in *Myxine*. The pattern of this ionic regulation does not resemble any of those found in the marine invertebrates (Robertson, 1949, 1953); sodium is present in higher concentration in the plasma, but all other ions (except bicarbonate and phosphate) are lower than those in the sea water. An osmotic gradient of at most 1–2 % will attract water plus salts to replace the fluid filtered off by the large glomeruli of the kidneys. The total filtration area in *Myxine* has been estimated by Nash (1931) to be greater than that of many marine teleosts and equal to that of many fresh-water ones (on the basis of an equal surface area of fish). Quantity and composition of urine have not yet been investigated, but it would seem that resorption and secretion of ions may well occur in the tubules, playing a part in the mechanism of ionic regulation.

In the lampreys that part of the life history spent in fresh water involves control of the salt content of the internal medium at levels far above that of the streams and lakes. Such control seems to be similar to that of the fresh-water teleosts and other actinopterygians, and presumably involves active uptake of ions from the dilute external medium to replace those lost in the urine, and lost from permeable body surfaces by diffusion. It has already been mentioned that the anadromous *Petromyzon marinus* and *Lampetra fluviatilis* caught in rivers are apparently unable to withstand transference to sea water; the physiological state of these lampreys, with their atrophied digestive tract, has deteriorated as far as osmotic regulation is concerned, and they die after spawning.

The analysis of the plasma of *L. fluviatilis* is similar to that of the Loch Lomond powan, except that most of the ions are in slightly lower concentration. No urea has been found in the blood of the lamprey, and only small amounts in *Myxine* comparable to those in teleosts, 122–255 mg./l. These amounts agree well with those found by Smith (1932), 150–280, but are only 3–7 % of those found by Borei (1935), whose results are probably erroneous. Needham (1938) states that the amount of urea found by Borei in *Myxine* is strictly comparable with that in fresh-water elasmobranchs, but he has confused urea with urea-nitrogen, and in fact the amount would be about half. Needham's further speculations based on Borei's high urea figure are that *Myxine*'s ancestors developed urea retention for marine life (like elasmobranchs), virtually lost this in a return to fresh water, and developed a new mechanism, salt retention, when they returned to the sea. Based, as they seem to be, on erroneous analyses, these ideas need not be considered seriously.

Lampetra fluviatilis has a lower bicarbonate content than *Coregonus clupeoides*,

6 as against 11 m.equiv./kg. water. Smith's figure of 4 m.equiv. for *Myxine glutinosa* (in Cole, 1940) also contrasts with the present value for *Muraena helena*—8 m.equiv. Perhaps these differences are related to the absence of calcareous structures in the cyclostomes, but this suggestion does not receive additional support from the figures of Fontaine & Boucher-Firly (1934) who find that *Petromyzon marinus* with 9 m.equiv. exceeds the average bicarbonate content of 3 m.equiv. found in two elasmobranchs. The latter, of course, have calcified cartilage in the vertebral column. In such comparisons more attention should be paid to the bicarbonate content of the external medium; in the cases of *Lampetra* and *Coregonus*, the waters were very soft and had bicarbonate concentrations of 0·1 and 0·2 m.equiv. respectively. One may assume 2–3 m.equiv. for the sea water of the French workers.

Fontaine (1932) claims a definite correlation between the phosphorus levels in the serum of marine bony fish, and those of the marine lamprey (in fresh water) and elasmobranchs, the values of the former being 2–3 times as high, with the lowest value (56 mg. P/l.) in the lamprey. Such a low value has not been found in the present work, where the levels of phosphorus in cyclostomes are three times that of Fontaine's figure for *Petromyzon*, *Myxine* with 175–206 and *Lampetra* with 207 mg. P/l. In fact these levels are slightly higher than those in *Coregonus* and *Muraena*, the two teleosts studied. The phosphorus level in the blood is probably influenced by the state of the phosphorus balance in muscle, as well as by the inorganic phosphate of bone. Unpublished data on the total phosphorus of *Myxine* muscle show it to be even greater than in *Muraena* muscle, 2·2 mg. compared with 1·8 mg./g. fresh weight.

Coregonus plasma with its pH of 7·5 compares with Loch Lomond water of pH 6·9. With one exception, this slightly alkaline reaction in the plasma agrees with the few values in the literature (e.g. Püschel's (1928) figure of 7·51 for heart blood in *Tinca*). Willmer (1934), however, found that the pH of the blood of freshwater fishes in acid or slightly acid river and swamp waters varied between 6·2 and 6·8. His results are rather doubtful since he used colorimetric methods. In the present work it was found that the apparent pH of *Coregonus* plasma as determined by adding bromo-thymol blue indicator was 6·5, whereas a value of 7·51 was obtained with the glass electrode. The error seemed to be a peculiarity of this indicator, as the apparent pH using phenol red was 7·4.

Table 6 sets out the relative ionic composition of the plasma of the various chordates investigated, based on a chloride value of 100. This ion balances 83–94% of the cations in all the animals except the lamprey, where the figure is 73%. Tunicate blood is seen to resemble sea water in total ionic concentration, and in relative composition including ratios of equivalents. The cyclostomes and teleosts are alike in showing a relative decrease in magnesium, which is chiefly responsible for the rise in the ratios of equivalents in the four species. Florkin (1949) has found that the ratio $Na+K/Ca+Mg$ is definitely greater in vertebrates compared with invertebrates, most values being above 11. This is confirmed in the analyses above. Out of thirty-seven marine invertebrate ratios (Robertson, 1949, 1953, and the two

tunicates) only two have exceeded 9·7, those of the lobsters *Nephrops* and *Homarus* with 11·5. The low magnesium content of the blood is chiefly responsible for the relatively high ionic ratios in lobsters and vertebrates.

Table 6. *Relative ionic composition of plasma of aquatic chordates*

	Total ions relative to sea water	Weight units (g.)						Equivalents		
		Na	K	Ca	Mg	Cl	SO_4	$\frac{Na+K}{Ca+Mg}$	$\frac{K}{Mg}$	$\frac{Ca}{Mg}$
Sea water	100	55·5	2·01	2·12	6·69	100	14·0	3·8	0·09	0·19
Phallusia	100	53·3	1·95	1·91	6·38	100	7·1	3·8	0·09	0·18
Salpa	100	54·4	2·22	1·99	6·20	100	8·8	4·0	0·11	0·19
Myxine	98	62·9	1·84	1·23	2·31	100	3·1	11·1	0·25	0·32
Lampetra	21*	80·9	3·69	2·32	1·51	100	7·7	15·1	0·76	0·93
Muraena	32	72·9	1·14	2·32	0·88	100	8·2	17·0	0·40	1·59
Coregonus	25*	75·7	3·48	2·50	0·96	100	5·1	16·6	1·13	1·58

* A sea water of 1135 mg. ions/kg. water has been taken for comparison in the case of these fresh-water animals. At 20° C. this sea water has a chlorinity of 19·00‰ and a chloride content of 19·68 g./kg. water.

The general resemblance in ionic composition of the plasmas of marine chordates to sea water (Table 6) is probably a reflexion of a certain degree of permeability of the gills and other surfaces to ions. Existing differences are presumably the result of active regulation by the boundary surfaces of the animals, aided in the cyclostomes and bony fishes by the kidneys. Similarity in relative ionic composition between *Lampetra* and *Coregonus* on the one hand, and the marine *Myxine* and *Muraena* on the other, falls in line with the view that the tissues of all the aquatic vertebrates are adapted to function in an internal medium of broadly the same relative composition as sea water. Active uptake of ions from fresh water by lampreys and fishes must therefore clearly differentiate between sodium and potassium, since the external medium has a low Na/K ratio (usually between 2 : 1 and 4 : 1 on a weight basis) in comparison to the high Na/K ratio maintained in the plasma. Krogh (1939) has indeed shown that the goldfish is unable to absorb actively the K^+ ions from solutions of potassium chloride, whereas it could take up the Cl^+ anion; it could, however, absorb both ions from a solution of sodium chloride.

Control of permeability to water is obvious in marine teleosts and in anadromous and fresh-water cyclostomes and fishes. It is perhaps not so obvious in myxinoids, but these animals may be very slightly hyperosmotic to sea water, ensuring intake of water, probably through the gills. If the excess of osmotic pressure in the blood, due to salts and colloids, is insufficient to counterbalance the hydrostatic pressure in the capillaries of the gills and any other water-permeable surfaces, then any water uptake must be active; precise data with which to settle these points are not available.

The striking divergence in osmoregulation between myxinoids and petromyzonts led Smith (1932) to suggest that these two groups lead back to a 'parting of the ways' in the evolution of body-fluid regulation. Myxinoids resemble the

lower marine chordates and other marine invertebrates in their osmoregulation, but differ from them in the pattern of ionic regulation. Alone among the aquatic vertebrates, this group maintains a salt content at least three times that characteristic of its related order, the Petromyzontia, and of all groups of true fishes. The problem here is whether this high salt content and practical osmotic equilibrium is a primary feature, being derived directly from marine chordate ancestors, or is secondarily derived by modification from fresh-water ancestors with a reduced salt content in their body fluids.

Prevalent opinion, as recorded in recent text-books, supports the belief that the Agnatha (including the ancestors of the cyclostomes) and the principal groups of fishes are of fresh-water origin. This is the view of Romer & Grove (1935), based on consideration of the American palaeontological evidence. However, fossil agnathans and primitive fishes are often found associated with eurypterids, and Ruedemann (1934) claims that in the shale beds of New York these arthropods are associated with marine faunal elements. Although eurypterids were fresh-water in habitat in the Carboniferous, he believes they inhabited lagoons and embayments of the marine littoral region in the Ordovician and Silurian periods.

The oldest known vertebrate remains, plates and scales of ostracoderms of Middle Ordovician age, are in sandstones of marine origin, as judged from the associated invertebrates (Bryant, 1936; Kirk, 1930). While Romer & Grove concede that the vertebrates in this Harding sandstone may have been estuarine, they take the view that these animals were inhabitants of fresh water in life. Their evidence for this latter hypothesis consists of the fragmentary nature of the fossils, and the apparent littoral nature of the deposit, completely equivocal evidence which they interpret one way. Much stronger evidence than 'the fragmentary nature of the fossils' must be provided before the obvious interpretation of the juxtaposition of scales and marine invertebrates is discarded for a less likely view.

It is by no means improbable, therefore, that the agnathans were a primitively marine group, secondarily spreading into fresh water, and that the ancestors of the modern myxinoids have always been marine. On this hypothesis, the acquisition of an inherited intensification of the processes of ion and water regulation in some cyclostomes resulted in a lower osmotic pressure of the blood, enabling members of what became the petromyzont branch to pass into fresh water. Whether the division of the cyclostomes into two orders took place recently (Goodrich, 1931; White, 1935) or in the Palaeozoic era (Stensiö, 1927, 1932) is obscure, since no near relatives of the living forms exist in the fossil record.

The alternative hypothesis of a fresh-water origin of the cyclostomes would suggest that the myxinoids were secondarily marine, and had lost to a great extent their presumed original osmotic independence of the environment. This view might be supported if any other cases were known of an unequivocal reversion of osmotic independence in other groups. In this connexion, examination of the body fluids of the marine coelacanths *Latimeria* and *Malania* (Smith, 1939, 1953) may be rewarding. Such features as the internal nares and lungs found in their fresh-water relatives have apparently been lost in these crossopterygians.

In support of this second hypothesis is the general theory of the evolution of the glomeruli of the kidney in fresh-water protovertebrates in response to a need for excreting large quantities of water (Smith, 1932; Marshall, 1934). Myxinoids with their glomerular kidneys would naturally fall into the category of animals with a fresh-water ancestry, if the postulates of this theory were rigidly applied. Now, the general findings of palaeontology, comparative morphology and physiology seem to fit this theory in a general way in the bony fishes, which seem to have been originally fresh-water in habitat. Marine teleosts have kidneys with a total filtration surface smaller than that of their fresh-water relatives (Nash, 1931), a finding in keeping with the reduction in water excretion by the kidneys, partly as a result of the reversed osmotic gradient with the environment. A few marine teleosts are aglomerular, a fact taken by Smith and Marshall as evidence that reduction and loss of glomeruli is taking place in the group as a whole.

Summarizing the data of Edwards (1928), Marshall & Smith (1930), and Nash (1931) for marine teleosts, twenty-nine families and forty genera are glomerular, five families and seven genera almost aglomerular, and ten families and fifteen genera wholly aglomerular. To this list Smith (1931) would add an aglomerular genus and two almost aglomerular families, each represented by a genus. These data do not altogether support current text-book statements of few glomeruli or absence of glomeruli in the marine teleosts. All the common 'successful' families, Clupeidae (herrings), Gadidae (cod, haddock, etc.), Pleuronectidae (plaice, etc.) and Scombridae (mackerel) are glomerular, and also the Muraenidae, to which *Muraena* belongs. While a certain reduction in glomeruli or in total filtration surface (volume times number of glomeruli per unit body surface) is in keeping with the changed osmotic gradient of the marine teleost compared with its fresh-water relative, this relationship is not evident in the elasmobranchs and marine cyclostomes, as the following facts show:

(*a*) Nash (1931) has estimated that the total filtration surface of the kidney in some *marine* elasmobranchs is of the same order as that in *fresh-water* teleosts. Here there is no relation of number of glomeruli with osmotic gradient, which is very slight in the elasmobranchs* and steep in the teleosts; the relation is between the amount of urine produced and the osmotic gradient, similar filtration surfaces producing large amounts of urine in the fresh-water teleosts and small amounts in the marine elasmobranchs.

(*b*) Marine teleosts and marine elasmobranchs with their very different total filtration surfaces (Nash) and osmotic gradients with sea water, features which might be expected to reduce the amount of urine in the first group compared with the second, yet produce about the same amount of urine per unit weight and time (Marshall, 1934; Krogh, 1939). The sea water which marine teleosts drink is apparently their chief source of water for the urine. Again there is no relationship

* The supposed hypertonicity of marine elasmobranchs and *Myxine* (Smith, 1932) depends on orthodox freezing-point determinations, subject to the difficulties and errors associated with measurements in colloid-containing solutions (e.g. Blanchard, 1940). Vapour-pressure measurements on *Scyllium* by Margaria (1931) indicate isosmoticity within 0·8 %.

between glomerular development and the quantity of water normally excreted, contrary to the thesis of Marshall & Smith (1930).

(*c*) *Myxine*, isosmotic with sea water or at most 2 % hyperosmotic, has a filtration surface larger than that of most of the twenty-five marine teleosts examined by Nash (1931), and almost equal to that of his fresh-water teleosts. The amount of urine produced by *Myxine* is not known, but it is highly improbable that it approaches that of fresh-water teleosts; yet the decreased or absent osmotic gradient has not reduced the filtration area of the myxinoid kidney.

Since the glomeruli of the myxinoid and elasmobranch kidney show no reduction despite a very small or virtually absent osmotic gradient, one of the principal theses of Marshall & Smith (1930) fails when applied to these groups.

The supposed fresh-water origin of the glomerulus rests on two postulates, the function of the glomerulus as a filter, and the alleged palaeontological evidence that the first vertebrates lived in fresh water (Marshall & Smith, 1930; Smith, 1932). The first postulate is probably correct, but filtration depends on hydrostatic pressure in the blood capillaries, not on any osmotic gradient. Mere existence of glomeruli in no degree supports a view that they necessarily had a fresh-water origin or are essential to a fresh-water existence. Indeed, certain aglomerular pipefishes are found in fresh water (Grafflin, 1937). The second postulate is incapable of proof, and probably incorrect, as the palaeontological evidence already discussed implies.

Krogh (1939) also doubts whether the first development of the glomerular kidney took place in fresh water. He has pointed out that the principle of the glomerular kidney is the same as that in many invertebrates, particularly in the decapod Crustacea. In these animals there appears to be filtration of a fluid under pressure with subsequent modification by reabsorption and secretion, and this may take place in *Carcinus* (Picken, 1936) and other genera living in sea water in which there may be no difference in osmotic pressure between the internal and external media. In these decapod crustaceans a 'urine' flow of about 50 ml./kg./24 hr., much greater than in marine elasmobranchs, is based on active uptake of ions by the gills, associated with uptake of water, which also seems to be an active process (Webb, 1940). There is therefore no need to invoke large osmotic differences as being a necessary stimulus to the development of the glomerulus, although these might subsequently determine the number of glomeruli, to some extent.

It is therefore concluded that the presence of glomeruli in *Myxine* in no way can decide whether the animals have had an early fresh-water or marine ancestry. On balance the writer takes the primary marine origin of the myxinoids to be the more probable, and suggests that the kidneys of the marine ancestors of these animals were glomerular, as are the kidneys of the present-day genera.

Regarding the evolutionary origin of the glomerulus, it is suggested that this structure may have developed in marine protovertebrates as an aid to flushing the excretory tubules when the coelome, with which the inner part of each tubule was once connected, ceased to be a cavity completely filled with fluid. The advantages of filtering blood with excretory substances collected from all parts of the body were

such that open peritoneal funnels or nephrostomes have disappeared in all fishes and higher groups, except in myxinoids, in some elasmobranchs, in the larvae of petromyzonts, dipnoans and holosteans, and in amphibians (Bles, 1898; Goodrich, 1930).

SUMMARY

1. Fairly complete analyses have been given of the blood of certain tunicates, cyclostomes, and bony fishes.

2. The plasma of the tunicates *Phallusia mammillata* and *Salpa maxima* closely resembles sea water in ionic composition, except for a very reduced sulphate content. Protein is less than 1 g./l.

3. The serum of *Myxine glutinosa* is isosmotic with sea water within about 1 %. Every ion is regulated; sodium and phosphate exceed their respective values in sea water, and the remaining ions are lower, especially sulphate and magnesium. Two values for urea are 122 and 255 mg./kg. water, the higher figure being about 0·3 % of the total concentration of ions and non-electrolytes.

4. The composition of the plasma of *Lampetra fluviatilis* from fresh water resembles that of *Coregonus clupeoides*, a fresh-water teleost. *Muraena helena*, a marine teleost, maintains a total concentration of ions about one-third that of sea water. It differs from fresh-water teleosts chiefly in maintaining much higher sodium and chloride concentrations.

5. The implications of the dichotomy in osmotic and ionic regulation of myxinoids and petromyzonts are considered, and also the glomerular nature of the cyclostome and fish kidney.

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THE MECHANICAL PROPERTIES OF THE CELL SURFACE

I. THE CELL ELASTIMETER

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(With Plate 10)

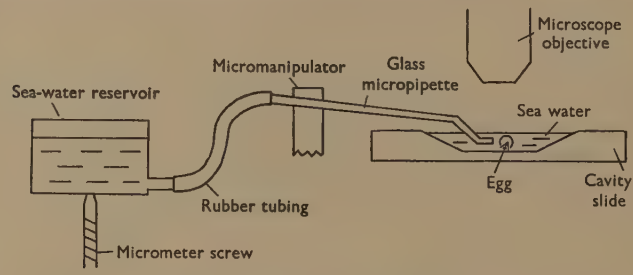
INTRODUCTION

In two recent papers (Mitchison, 1952; Swann, 1952) we have put forward a new theory of cell division, based on the idea of expansion in the cell membrane. Proof or disproof of this theory is only possible from a detailed knowledge of the tension and other mechanical properties of the cell surface at the time of cleavage. The experiments described in this and the following papers were started with the object of providing such information.

Investigating the mechanical properties of the surface of so small an object as a cell presents considerable difficulties. It would hardly be desirable, even if it were possible, to isolate a piece of cell membrane. It is necessary, therefore, to deform the intact cell in some way, such that the deformation and the deforming force can both be measured. Various attempts have been made in the past to do this, mainly by direct mechanical compression, by stretching between needles or in a centrifugal field, or by observing the degree of flattening under gravity. References are given by Harvey & Danielli (1938). The most satisfactory experiments were those of Cole (1932), who devised a delicate and ingenious method of compressing sea-urchin eggs with a minute gold beam. These experiments gave an unequivocal proof that the cell surface behaves like an ordinary elastic material whose tension increases with extension, rather than an interface whose surface tension is independent of the extension. Cole, however, also extrapolated the results from small compressions to give a figure of 40 dynes/cm.^{2} for the internal pressure of the undeformed egg (equivalent to a 'surface tension' of 0·08 dyne/cm.). For reasons given in the second paper of this series (Mitchison & Swann, 1954), we believe the method of extrapolation to be unjustified, and the results that follow from it to be of doubtful value. There is also a general objection to Cole's method: reliable readings can only be obtained with large compressions of the egg, and such deformations are liable to measure not only the resistance of the egg surface, but of any internal structures as well. At the stage of the sperm aster or the amphiaster this may well produce confusing results. It was with the object of overcoming this difficulty that we developed a new and more sensitive instrument, which we have called the 'cell elastimeter'.

THE CELL ELASTIMETER

The apparatus is in essence extremely simple. It consists of a glass micropipette filled with water and connected by rubber tubing to a small movable reservoir of water. Using a microscope and a micromanipulator the pipette is brought up to a cell (eggs of various sea urchins in the present experiments) and the reservoir is then lowered slightly with a micrometer screw. This creates a small suction which draws the egg on to the end of the pipette, where it makes a seal, with the surface of the egg bulging slightly up the tube (Text-fig. 1; Pl. 10, fig. 1). If the reservoir is then lowered again, the cell surface bulges progressively further up the pipette. The deformation or degree of bulging is measured directly under the microscope; the negative hydrostatic pressure or suction is measured on the micrometer screw.



Text-fig. 1. General arrangements of the cell elastimeter (not to scale).

In the first version of the apparatus the reservoir was fixed, with one tube leading to the micropipette, and another to a hypodermic syringe fitted with a screw thread. The level of water in the reservoir was varied by moving the syringe, and measured by means of a float and optical lever. But this arrangement proved cumbersome and needlessly accurate. In the final version (Pl. 10, fig. 2) the reservoir, connected by a single tube to the pipette, is mounted at the end of a strip of spring steel, and can be moved up and down by a micrometer, with a large disk on it graduated in hundredths of a millimetre. The micrometer is also geared to a revolution-counter measuring tenths of a millimetre. The micrometer and the reservoir are mounted on three screw legs so that a rough height adjustment can be made.

The construction of the micropipette presents various problems. There is first of all the question of diameter. This must clearly be less than the diameter of the egg (100 μ), which would otherwise be sucked right up the tube. On the other hand, the smaller the diameter of the pipette, the greater the difficulty of measuring accurately the degree of bulging of the cell surface, and of making the initial equilibration (see p. 445). After some trials, pipettes of about 50μ diameter were found to be best for working with sea-urchin eggs.

There is, secondly, the question of getting pipettes with clean straight ends, so that eggs can seal without difficulty, and the zero point for readings of deformation can be determined precisely. To make such pipettes, glass tubing is drawn out

roughly and bent so as to reach down on to a microscope slide. The end is then drawn out by hand to a fine point with a considerable taper, and broken off with a microforge in the manner described by Fonbrune (1949, p. 42). About one break in five is square and clean enough to be used. The internal diameter is determined by immersing the point in benzene or some other volatile medium of fairly high refractive index, and measuring with a micrometer eyepiece under the microscope. Pyrex or Jena glass tubing gives a higher proportion of clean straight ends than soft glass.

There is one further difficulty to be overcome with the pipette, namely the making of a constriction a short distance ($\frac{1}{2}$–1 mm.) behind the end. The object of this is simply to damp down the flow in and out of the pipette. With a diameter of 50 μ, the flow is sufficiently rapid for chance vibrations to blow the egg off the end, and, worse still, to draw it back again violently. The egg then usually bursts and spreads itself over the end of the pipette, when it can only be removed by drastic treatment with cleaning solution. A constriction of 30 μ diameter or so gets over this difficulty, while a narrower constriction retards the flow so much that the process of equilibration, described in the next paragraph, becomes very difficult (the rate of flow in tubes is proportional to the fourth power of the diameter). The constriction was also made on the microforge in the manner described by Fonbrune (1949, p. 61).*

A satisfactory pipette having been made, filled with sea water by means of a suction pump and connected to the reservoir by a short length of rubber tubing, it is mounted on a micromanipulator (in our experiments a Zeiss Peterfi or Zeiss 'gliding' model) and its point dipped in about 1 ml. of sea water and eggs on a large cavity slide. It is then necessary to equalize the water levels of the slide and the reservoir. This is best done by introducing a small quantity of milk. If the reservoir level is too low, the minute oil droplets of the milk are seen to flow up the tube: if the reservoir is too high, an outflow from the tube is immediately apparent. Rough equalization is achieved by screwing the legs of the elastimeter up or down, or by altering the amount of water in the reservoir. The final equalization is made with the micrometer screw and can be very precise indeed; a movement of $\frac{1}{100}$th mm. or less is enough to reverse the direction of flow in the pipette.

In the course of a few minutes, the fat droplets rise up out of the focal plane of microscope, and leave the field clear for observation. If they are needed later for re-equilibrating, the water on the slide can be stirred up with a bulb pipette. It should be added that the small amount of milk has no adverse effect on the eggs, which can develop satisfactorily in concentrations of milk in sea water as high as 25 %. In the course of a long series of experiments, the equilibration point is liable to drift slowly as a result of the different rates of evaporation from the water surfaces on the slide and the reservoir. Fresh equilibrations must therefore be made from time to time.

Equilibration is carried out with the tip of the pipette just clear of the surface of the slide. An egg can then be manoeuvred up to the mouth of the pipette using the

* In some laboratories (e.g. Millport) where there is little vibration, a constriction may not be necessary.

stage controls of the microscope. The first reading is made with the minimum negative pressure necessary for getting the egg to seal on to the pipette—usually about $\frac{1}{10}$th or $\frac{1}{20}$th mm of sea water. Successive readings are then made with greater pressures, up to the point when the bulge becomes a hemisphere. Between six and eight readings are made on each egg, and this takes about 2 min.

The eggs must be free of any outer coatings. In this case of unfertilized eggs this means removing the jelly. With *Psammechinus miliaris* at Millport this can be done simply by repeated washings in sea water. With the Mediterranean species at Naples (*P. microtuberculatus*, *Paracentrotus lividus*, *Arbacia lixula* and *Sphaerechinus granularis*) treatment with acid sea water is necessary. Two minutes in a mixture of 100 ml. sea water, and 3 ml. of M/10 HCl is usually sufficient, but the eggs of *Sphaerechinus* occasionally need longer. In the case of fertilized eggs, the fertilization membrane must be removed. This can be done most conveniently by passing the eggs through fine bolting silk about 2 min. after fertilization.

The degree of bulging up the pipette is read directly with an eyepiece micrometer to an accuracy of about $\pm 0{\cdot}7\ \mu$. It is convenient to use a binocular microscope, with a low-power eyepiece ($\times 5$) in one socket, for finding eggs and getting them to the mouth of the pipette, and a high-power eyepiece ($\times 15$) and micrometer in the other socket, for measuring the deformation. The micrometer plate is rotated so that the scale lies along the pipette axis, and the micromanipulator is adjusted to bring one of the major divisions, or the end of the scale, level with the end of the pipette (see Pl. 10, fig. 1).

After a series of readings on an egg, the reservoir is raised high enough to give a slight positive pressure, when the egg usually falls off the pipette. If it shows any tendency to stick, it can be jerked off by tapping the bench, or blown off forcibly by pinching the rubber tubing connecting the pipette to the reservoir.

THE BEHAVIOUR OF EGGS ON THE PIPETTE

A series of readings of deformation for increasing negative hydrostatic pressures gives a plot that is nearly linear for both unfertilized and fertilized eggs (Text-fig. 2). The slope, however, varies with the different stages of development. It depends also on the relative sizes of the egg and the pipette, and to some extent on the speed at which readings are taken. For reasons explained on p. 453, we have called the slope of the pressure-deformation curve the 'stiffness'.

The egg surface is not a purely elastic system; there is an appreciable viscous component, and the membrane always takes a little time to reach a constant deformation. This is evident from the graphs in Text-figs. 3–5, which show the pressure-deformation curves for unfertilized eggs of *Psammechinus miliaris* with a 41μ pipette, for readings taken at different speeds. In Text-fig. 3 the readings were taken at 10 sec. intervals and give a slope or stiffness of 17·5 (dynes/cm.^{2}/μ deformation). In Text-fig. 4 the readings were taken at 1 min. intervals and give a stiffness of 14·2. In Text-fig. 5 the deformation was allowed to reach a constant value; this takes several minutes, and gives a stiffness of 11·6. There is thus a factor of about 1·5

between the stiffnesses given by slow and fast readings. In practice it is not feasible to wait for the deformation to come to rest, and readings were always taken at about 15 sec. intervals. It follows that the stiffnesses obtained were always on the high side.

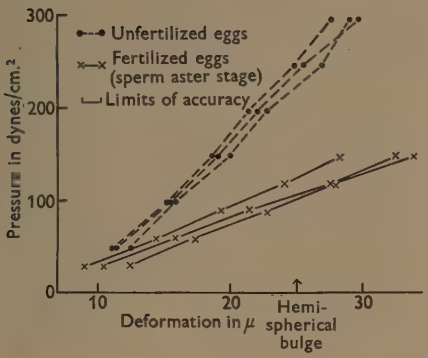


Fig. 2

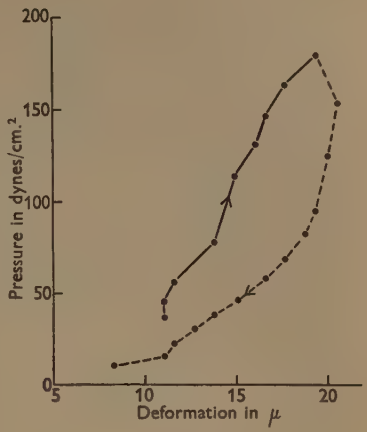


Fig. 3

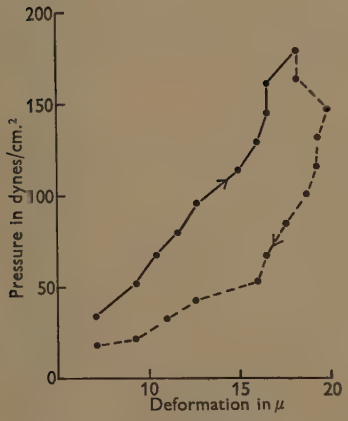


Fig. 4

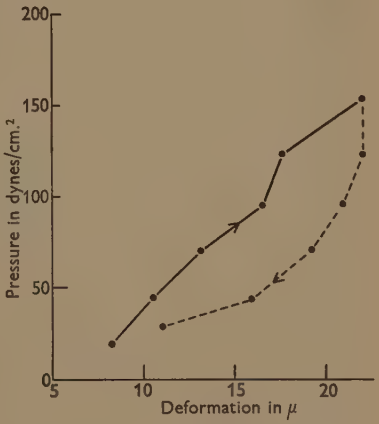


Fig. 5

Text-fig. 2. Pressure-deformation curves for individual eggs of *Psammechinus microtuberculatus* (50 μ pipette).

Text-figs. 3–5. Pressure-deformation curves for eggs of the same batch; increasing and decreasing negative hydrostatic pressures; varying speeds of measurement. Unfertilized eggs of *P. miliaris*; 41 μ pipette. Text-fig. 3. Quick. 10 sec. intervals between readings. Text-fig. 4. Slow. 1 min. intervals between readings. Text-fig. 5. Membrane allowed to come to rest between readings.

This viscous behaviour, or 'creep', is presumably due primarily to true viscous flow within the cell surface. It may, however, also be due to friction, hindering the free movement of the cell surface over the edge of the pipette. It should be pointed out that the surface does visibly slip over the edge of the pipette, so that the whole surface of the egg is stretched, and not merely the region within the pipette. In consequence, the overall degree of stretch is small; for a 100 μ egg with a hemispherical bulge in a 50 μ pipette, the surface area only increases by about 5 %.

Occasionally, however, the surface sticks to the edge of the pipette, especially in the case of *Arbacia* eggs. Under these conditions the mechanical situation is entirely different, since the tension in the membrane is not distributed evenly over the surface. For this reason, no measurements were made on eggs which showed a tendency to stick.

In view of the 'creep' mentioned above, it might be expected that the curves of deformation for increasing and decreasing negative hydrostatic pressures would not be the same. There is, in fact, a marked hysteresis, and the deformation for a given pressure is always greater on the decreasing curve (Text-figs. 3–5). This may be due partially to a viscous component in the membrane, but however slowly the readings are taken the upward and downward curves never coincide, suggesting that the main cause is friction between the cell surface and the edge of the pipette. The effect can best be visualized by taking a rubber band, holding one edge fixed on the top of a table, and pulling the other end downwards over the edge. As the free end is pulled down, the horizontal limb can be seen to extend steadily. If, however, the band is then slowly released, the horizontal limb does not start to shorten until there has been a substantial shortening in the vertical limb. In view of this frictional effect, and the consequent hysteresis, readings were normally taken only with increasing pressures.

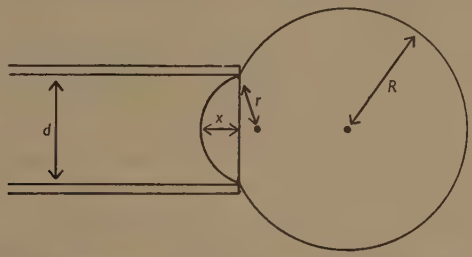
Where there is no question of friction at the edge of the pipette, a gradual 'creep' must of course be due solely to a viscous component in the surface or interior of the egg. This is the case when an egg is deformed on a pipette, and then suddenly knocked off. The retraction of the bulge takes about 10 sec.

Besides an element of viscous flow in the cell surface, there appears also to be something in the nature of a 'yield point'. Sometimes, in the course of a series of readings, the membrane 'gives way' suddenly, and the bulge moves rapidly right up the pipette. There is no cytolysis, however, and the bulge retracts if the egg is removed from the pipette. This 'yield point' is only reached in cleaving eggs, or eggs at low temperatures, and then only occasionally. Whether this phenomenon represents a genuine yield point of the structural membrane (as opposed to the permeability membrane, which presumably remains intact) is uncertain. It may simply be the result of changes in the elastic modulus of the membrane, with increasing extensions. The 'yield point' of the egg can, in fact, be simulated if a bulge is sucked out of a balloon that has been stretched nearly but not quite to the point when its modulus starts to decrease markedly. At first the bulge comes out slowly, but then quite suddenly it moves rapidly right up the tube before coming to rest again.

THE PROBLEM OF ANALYSIS

The cell elastimeter provides information on the extent to which the cell surface is drawn up the pipette by a given negative hydrostatic pressure. The conversion of this data to information on the mechanical properties of the cell surface presents considerable difficulties.

The most obvious method of analysis is in terms of simple surface tension. However, it has long been realized that the cell surface is not a simple interface but a definite structure with elastic properties. This point was emphasized by Cole (1932) in his experiments on compressing eggs with a gold beam. It is, nevertheless, perfectly legitimate to treat the problem using the surface tension formulae, provided the membrane is sufficiently thin for considerations of rigidity not to enter in. It should be pointed out, however, that if the system was really one involving simple surface tension, the figures for surface tension would come out constant for all deformations, whereas with an elastic system, the 'surface tensions' will be higher the greater the deformation. The tension at nil deformation can then only be deduced by extrapolation backwards from a series of points, a procedure which is liable to serious errors.



Text-fig. 6. Diagram for the analysis of the cell elastimeter problem in surface-tension terms.

The analysis of the problem in terms of surface tension is quite simple. If P is the (negative) hydrostatic pressure in the pipette, T is the surface tension of the egg and the other symbols are as shown in Text-fig. 6, then

$$P=2T\left(\frac{1}{r}-\frac{1}{R}\right) \quad \text{and} \quad r^2=(\tfrac{1}{2}d)^2+(r-x)^2,$$

so that

$$P=2T\left(\frac{2x}{x^2+\frac{1}{4}d^2}-\frac{1}{R}\right).$$

This curve is plotted in Text-fig. 7, for values of x up to $\frac{1}{2}d$ (hemispherical bulge), with $d=50\ \mu$ and $R=100\ \mu$, and arbitrary values for P and T. It should be compared with the curves actually obtained on eggs with the cell elastimeter (Text-fig. 2).

It will be apparent at once that whereas the theoretical curve is markedly convex, the experimental curves are, to a close approximation, straight lines. The cell surface is evidently not behaving as though it was a simple interface. This is not

surprising since there is every reason to suppose that the egg is a hollow elastic sphere; but in fact *thin-walled* elastic spheres normally do behave as though they had a simple surface tension, because although the true tension or force per unit cross-sectional area goes up during extension, the cross-sectional area itself goes down. A rubber balloon, for instance, behaves over quite a wide range of inflations as though it had a constant surface tension; as a result, model experiments with a tube sucking a bulge out of a rubber balloon (see next section) give marked convex curves very similar to the theoretical curve for simple surface tension (Text-fig. 8). In short, the egg is not behaving like a drop of liquid, nor is it behaving like a thin-walled elastic sphere.

This conclusion led us to question the legitimacy of treating the egg in surface tension terms, even recognizing that it may have elastic properties. The most likely explanation seemed to us that the cell surface is not thin at all, but relatively thick, so that the resistance it offers to deformation is not merely due to stretching, but to bending as well. It seemed possible, in fact, that the cell is not so much like a rubber balloon, as like a tennis ball, which by virtue of its thick wall has a certain rigidity, and does not collapse even when the tension in its wall is released by puncturing it.

This is not altogether an unexpected conclusion; there is general agreement that the cell membrane or cortex is fairly thick, and the work of Mitchison (1955) shows that it measures about 1·5 μ across. The essential step is simply the realization that a 1·5 μ membrane round a cell of 100 μ diameter is too thick for the surface-tension formulae to be valid.

Unfortunately, analysis of the deformation of a thick-walled sphere, whether or not it has an overall tension in its walls, is mathematically quite intractable. The only way to examine this hypothesis, therefore, is by means of model experiments; that is to say, by using rubber balls with known Young's modulus, wall thickness, and internal pressure, and sucking bulges out of them with measured negative pressures. Such methods are, of course, normal engineering practice. The results of experiments of this sort are given in the next section.

MODEL EXPERIMENTS

The validity of examining a problem in the deformation of cells by studying the deformation of rubber balls seems doubtful at first sight. The justification for such a procedure comes from dimensional analysis, which shows that if the ratios of cell diameter, cell-wall thickness, pipette diameter and deformation are preserved, the negative pressure in the pipette and the positive pressure in the ball will vary linearly with the elastic (Young's) modulus of the ball. In other words, if the model is a precise scaling-up of the elastimeter, and the suction required for a given degree of deformation is n times as great as with the elastimeter, then the internal pressure of the ball and the modulus will both be n times as great as in the cell. The detailed dimensional analysis, for which we are indebted to Dr D. G. Ashwell of University College, Cardiff, is given as an Appendix.

The model experiments were scaled up 1000 times. In place of the cells (100 μ)

were rubber balls of 100 mm. diameter. In place of the pipettes were open-ended glass tubes of various diameters. The rubber balls were made for us by Rubber Technical Developments Ltd., Welwyn, out of latex, and were of various wall thicknesses from 0·8 to 18 mm. Each ball had a stalk so that it could be inflated with air or water to any desired pressure. The open-ended tubes or pipettes were connected to a hand vacuum pump and a mercury column. To get a satisfactory seal between the ball and the pipette, the ball had to be greased. The deformations produced were read with a telescope.

For the sake of completeness, some experiments were also done with flat sheets of various thicknesses, and with a solid ball. These were made of special low modulus rubber, in order to give greater deformation for a given suction. A solid ball of latex rubber can scarcely be deformed at all under the conditions of these experiments, even by a complete vacuum.

The use of rubber as a material requires some explanation. It is of course very convenient. It is also a high polymer substance containing long chain molecules, and likely therefore to resemble the cell surface in its general behaviour. The Young's modulus of rubber varies with large extensions, but over short ranges it obeys Hooke's law (i.e. gives a constant modulus) to a fairly close approximation. The maximum linear extensions involved in elastimeter experiments are only of the order of 2 or 3 %, and the Young's modulus of the different balls was therefore measured for this particular range. In presenting the results, all the figures from model experiments have been scaled to a Young's modulus of 10^7 dynes/cm.^{2}. The actual modulus of the latex balls was a little higher than this; the modulus of the soft rubber was about 10^6 dynes/cm.^{2}.

For all the hollow balls and all the flat sheets, with 'pipettes' between 35 and 61 mm. and over a considerable range of internal pressures, the curves of deformation against suction turn out to be straight lines; at the higher pressures, however, the curves tend to become convex (Text-fig. 9). The curves for rubber balloons (unstretched wall thickness about 0·25 mm.), as already mentioned, are convex (Text-fig. 8). The curves for the solid ball are concave (Text-fig. 10). There seems little doubt, therefore, that the hypothesis of a thick-walled cell, resisting deformation at least partially by virtue of rigidity, is essentially right.

One difficulty however remains: though a ball or a cell may resist deformation by virtue of rigidity, it may also have an internal pressure and therefore an overall tension contributing to its resistance to deformation. Over a wide range these two factors combine to give linear pressure-deformation curves, so that any given slope or stiffness can be the product of a high modulus and nil tension or a lower modulus and a higher tension. It is not in fact possible to disentangle the two factors directly, though in the other papers of this series an effort has been made to do so by indirect means.

For the purpose of analysing the mechanical properties of the sea-urchin egg membrane, only a limited amount of information from model experiments is required. For the sake of completeness, however, and because of further work planned on other material, we have included fairly full information on balls of

various wall thicknesses, at various internal pressures, using various sizes of 'pipette'. We have also included data on flat sheets of various thicknesses. This information is summarized in Text-figs. 11–15.

Text-figs. 11 and 12 show the effect of wall thickness on resistance to deformation, which is perhaps less pronounced than might have been expected. Text-fig. 13 shows the effect of the thickness of a flat sheet on resistance to deformation, which is

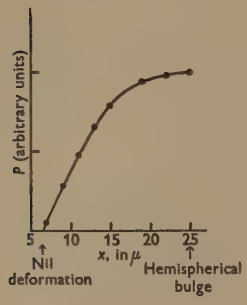


Fig. 7

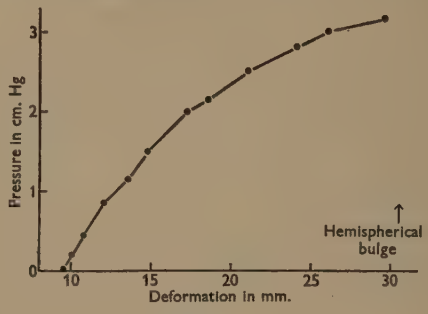


Fig. 8

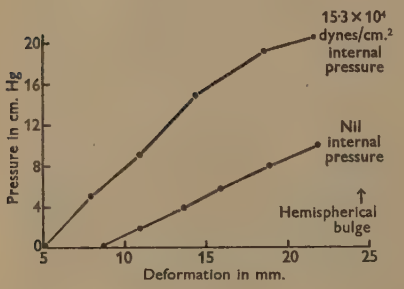


Fig. 9

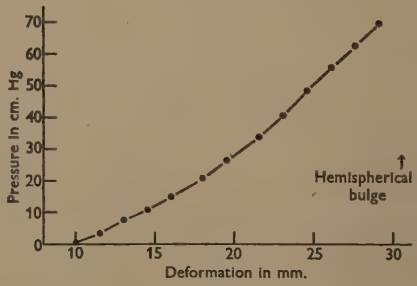


Fig. 10

Text-fig. 7. Theoretical pressure-deformation curve for pure surface tension. (50 μ pipette, 100 μ egg.)
Text-fig. 8. Pressure-deformation curve for a thin-walled inflated rubber balloon; 100 mm. diameter; unstretched wall thickness 0·25 mm.; 61 mm. pipette.
Text-fig. 9. Pressure-deformation curves for a rubber ball at different internal pressures; 100 mm. diameter; 1·5 mm. wall thickness; 49·5 mm. pipette.
Text-fig. 10. Pressure-deformation curve for a solid rubber ball; 100 mm. diameter; 61 mm. pipette.

more marked than is the case with balls. Text-fig. 14 shows the effect of internal pressure on the resistance to deformation of balls of different wall thicknesses, while Text-fig. 15 shows the effect of pipette diameter on a ball of 1·6 mm. wall thickness, at various internal pressures. The moduli have been corrected in every case to 10^7 dynes/cm.^{2}, and in Text-fig. 15 corrections have been made to the stiffness values to allow for the increase in diameter of the balls on inflation.

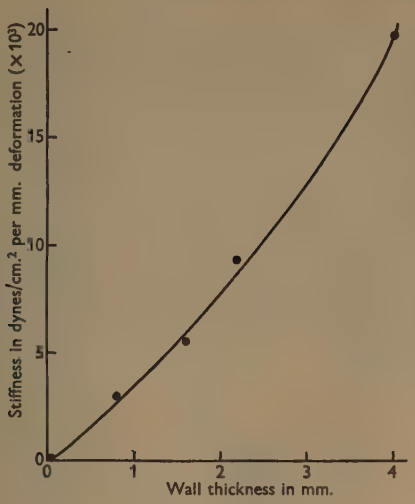


Fig. 11

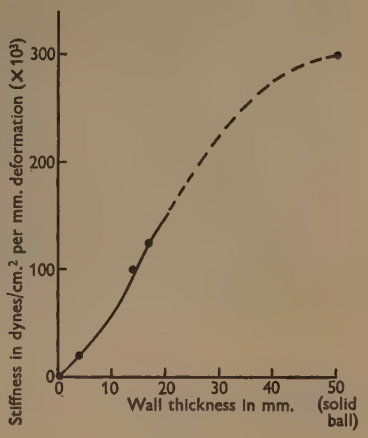


Fig. 12

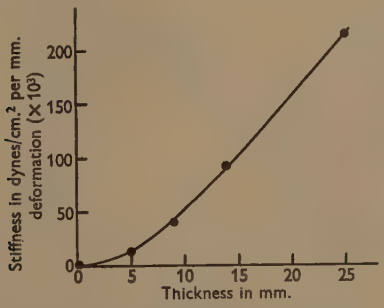


Fig. 13

Text-fig. 11. Stiffness as a function of wall thickness for rubber balls; 100 mm. diameter; 61 mm. pipette; nil internal pressure; Young's modulus values corrected to 10^7 dynes/cm.^{2}.

Text-fig. 12. As Text-fig. 11.

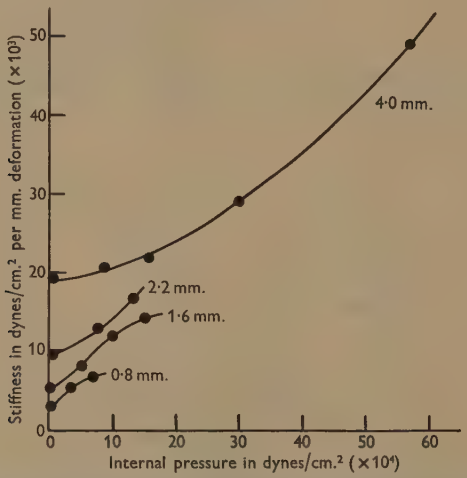
Text-fig. 13. Stiffness as a function of thickness for flat rubber sheets. 61 mm. pipette; Young's modulus values corrected to 10^7 dynes/cm.^{2}.

DETAILED ANALYSIS OF THE CELL ELASTIMETER RESULTS

The cell elastimeter measurements give a series of points on a pressure-deformation graph which happen to fall, rather conveniently, on a straight line. We have called the slope of this line the 'stiffness' of the membrane (measured in dynes/cm.^{2}/μ deformation). As will become apparent, stiffness is proportional, to a close approximation, to the Young's modulus of the membrane. We have, however, deliberately avoided using such terms as 'elasticity' or 'rigidity' for the slope of the curve in order to avoid confusion with the true moduli. Elasticity is in any

case an unsatisfactory word, since it is popularly assumed that rubber, for instance, is more elastic than steel, whereas its Young's modulus is actually much lower.

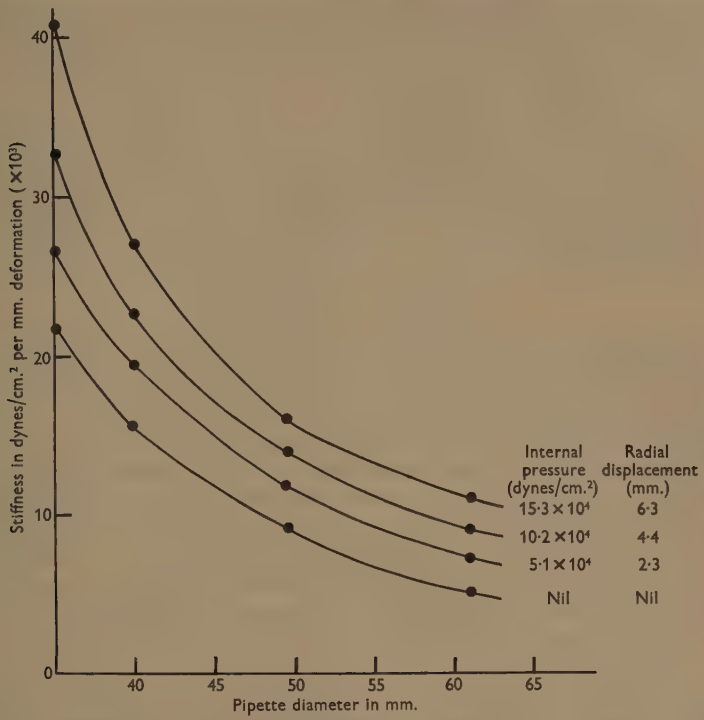
Stiffness depends of course on the elastic properties of the membrane. A full definition of the elastic behaviour of an isotropic solid, however, requires a knowledge of two of the following four variables: Young's modulus, the rigidity or shear modulus, the bulk modulus and Poisson's ratio. No doubt all of these are involved to some extent with the cell elastimeter, but since the problem is essentially one of bending and stretching, by far the most important variable is Young's modulus. Determining the absolute values of this modulus is done by scaling down from model experiments in the manner described below; but it should be emphasized that the procedure assumes that the membrane resembles rubber both in having the same Poisson's ratio, and the same relative values for the different moduli. The errors involved in this assumption cannot however be very large.



Text-fig. 14. Stiffness as a function of internal pressure, for rubber balls of various wall thicknesses; 100 mm. diameter; 61 mm. pipette; Young's modulus values corrected to 10^7 dynes/cm.^{2}; stiffness values are not corrected for radial displacement.

The first problem in analysing the cell elastimeter results is allowing for the fact that the eggs and the pipettes varied in size. This involves correcting the original 'uncorrected stiffness' figures, for a standard condition of a 50 μ pipette and a 100 μ egg. Suppose that a certain egg, of diameter $D\,\mu$ and a pipette of diameter $d\,\mu$ give an observed stiffness of s dynes/cm.^{2}/μ deformation. We must first correct s to allow for the difference between $D\,\mu$ and the standard egg size of 100 μ. This we can do by imagining both D and d to be multiplied by the ratio $100/D$. The egg diameter would become 100 μ, the pipette diameter would become $(100d/D)$ μ, and the stiffness (being inversely proportional to the observed deformation caused by a given applied pressure) would become $sD/100$ dynes/cm.^{2}/μ deformation. We

must now apply a second correction to allow for the fact that the new pipette diameter, $(100d/D)\,\mu$, may be different from the standard pipette diameter of $50\,\mu$. This we can do by using Text-fig. 15. If, for example, $D = 90\,\mu$ and $d = 40\,\mu$, so that the corrected pipette diameter is $100 \times 40/90 = 44\,\mu$, and if we assume nil



Text-fig. 15. Stiffness as a function of pipette diameter, at various internal pressures, for a rubber ball; 100 mm. diameter; 1·6 mm. wall thickness; Young's modulus corrected to 10^7 dynes/cm.^{2}; stiffness values corrected for radial displacement.

internal pressure, we find from the graph that altering the pipette diameter from the non-standard 44 units to the standard 50 units would multiply the stiffness by the ratio

$$\frac{\text{stiffness at 50 units diameter}}{\text{stiffness at 44 units diameter}} = \frac{9 \times 10^3}{12 \times 10^3}.$$

Thus applying both corrections, we find that an observed stiffness s, of a cell of diameter $D\mu$, used with a pipette of diameter $d\mu$, when corrected to the standard egg and pipette sizes, becomes

$$\frac{sD}{100} \times \frac{\text{stiffness for model with pipette diameter 50 mm.}}{\text{stiffness for model with pipette diameter } 100d/D \text{ mm.}}.$$

So far it has been assumed that the stiffness for the standard diameter and corrected diameter model pipette should be taken from the nil pressure curve of Text-fig. 15.

Strictly speaking, this is not justified; but, since all the curves are of very similar form, any error that may be introduced is certainly not serious.

The corrected stiffness figures for different eggs and different pipettes are of course all directly comparable. It remains, however, to extract from them information about Young's modulus and internal pressure. This can also be done with the help of the data of Text-fig. 15. It will be apparent from these curves, that any given stiffness can be the result of one modulus with nil internal pressure or another, lower, modulus with a higher internal pressure.

The case of nil internal pressure can be worked out direct from the lowest curve of Text-fig. 15. Suppose that a batch of eggs gives a corrected stiffness of 9 dynes/cm.^{2} (this is in fact about the figure for unfertilized eggs). The stiffness for the corresponding rubber ball of modulus 10^7 dynes/cm.^{2} with nil internal pressure, and using a 50 mm. pipette, is 9×10^3 dynes/cm.^{2} per mm. deflation. The modulus of the eggs is therefore

$$10^7 \times \frac{9}{9 \times 10^3} = 10^4 \text{ dynes/cm.}^2.$$

The alternative solutions, for a lower modulus and a higher tension, require more elaborate calculation, and they must be determined by constructing, from the information of Text-fig. 15, a fresh graph, which is given in Text-fig. 16. In this graph the elastic modulus and the internal pressure are plotted as a function of radial displacement, i.e. linear increase in radius. The existence of an internal pressure implies of course that the cell membrane is somewhat stretched, and the radial displacement is simply a measure of this degree of stretch.

Text-fig. 16 is derived from Text-fig. 15 in the following way. It is worked out for a corrected stiffness of 1 dyne/cm.$^2/\mu$ deformation, using a 50 μ pipette on a 100 μ egg. The figures for nil displacement in the egg are calculated direct from the lowest curve of Text-fig. 15 as before, i.e.

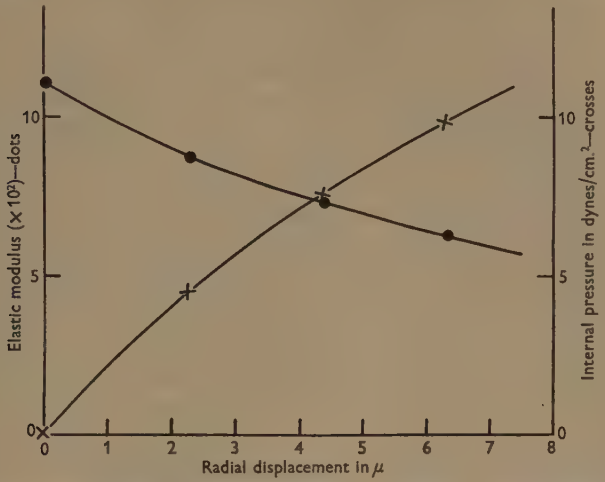
$$\text{modulus} = 10^7 \times \frac{1}{9 \times 10^3} = 11{\cdot}1 \times 10^2 \text{ dynes/cm.}^2 \text{ and nil internal pressure.}$$

A radial displacement of 2·3 mm. is associated in the model with an internal pressure of $5{\cdot}1 \times 10^4$ dynes/cm.^{2}, and gives a stiffness of $11{\cdot}5 \times 10^3$. The modulus for a 2·3 μ radial displacement in the egg must therefore be $10^7 \times \frac{1}{11{\cdot}5 \times 10^3} = 8{\cdot}7 \times 10^2$, and the internal pressure $5{\cdot}1 \times 10^4 \times \frac{1}{11{\cdot}5 \times 10^3} = 4{\cdot}44$ dynes/cm.^{2}. Similar calculations are made for the other radial displacements.

The results are plotted in Text-fig. 16, and give the different values of modulus and internal pressure which will produce a stiffness of 1 dyne/cm.$^2/\mu$ deformation. The figures can be multiplied up directly for measured figures of actual stiffness.

If the radial displacement or degree of stretch of the egg membrane were known, limits could be set to the values for modulus and internal pressure, and it will be seen from the next paper that there is reason to suppose that the radial displacement is not greater than 7 μ. Since the corrected stiffness for the unfertilized egg is

about 9 dynes/cm.^{2}/μ deformation, Young's modulus must therefore lie between 1×10^4 and $0{\cdot}54 \times 10^4$ dynes/cm.^{2}, and the internal pressure between 0 and 95 dynes/cm.^{2}.



Text-fig. 16. Young's modulus and internal pressure as a function of radial displacement for a 100 μ egg; 1·6 μ membrane thickness and 50 μ pipette, for a corrected stiffness of 1 dyne/cm.^{2}/μ deformation.

If there were no internal pressure in the egg, the membrane's resistance to small deformations could only be due to its rigidity or resistance to bending. If, on the other hand, there were an internal pressure, and hence an overall stretch in the membrane, there would be superimposed on the resistance to bending, a resistance to further stretching. In the light of the arguments above, it is of some interest to know the possible relative contributions of these two effects. A rough idea can be gained by comparing the observed stiffnesses of a model with nil internal pressure, and a model with an internal pressure corresponding to the maximum radial displacement (7 mm.). For a 50 mm. pipette it will be seen from Text-fig. 15 that the ratio is about 9/16. It follows that at least 55% (and perhaps 100%) of a cell's resistance to deformation must be due to the inherent rigidity of the membrane. The dangers of analysing the mechanical properties of the cell surface in terms that neglect this rigidity need no further emphasis.

NOTE ON RADIAL DISPLACEMENT

In the case of model experiments, radial displacement can of course be measured directly for different internal pressures. Alternatively, it can be calculated from the following formula, taken from Roark (1938):

$$P = \frac{2tdE}{R^2(1-\sigma)},$$

where P = internal pressure, t = membrane thickness, d = radial displacement, E = elastic (Young's) modulus, R = radius of sphere, σ = Poisson's ratio. Poisson's ratio for rubbery materials is usually about 0·5.

SUMMARY

1. A new instrument for measuring the mechanical properties of the cell surface is described. It consists of a micropipette connected to a moveable reservoir of water. The tip of the pipette is brought up to a cell and a bulge sucked out of the surface by lowering the reservoir.

2. A plot of deformation (i.e. degree of bulging) against negative hydrostatic pressure gives a straight line for the sea-urchin egg at all stages. We have called the slope of this line the 'stiffness' of the cell membrane; it varies with the stage of the egg, the size of the pipette and the speed at which readings are taken.

3. A comparison of the straight-line plots actually obtained with the curves to be expected from pure surface tension or from a thin-walled elastic sphere, leaves no doubt that the cell membrane is not behaving like either of these systems. It is concluded that the membrane is sufficiently thick to resist deformation by virtue of its own rigidity, resembling therefore a tennis ball rather than a rubber balloon or a fluid drop.

4. An analysis of the problem in terms of a thick membrane is mathematically intractable, and is only possible by means of models. Experiments, using a large-scale pipette to suck bulges out of rubber balls, show that over a considerable range of wall thicknesses, the pressure-deformation curves are in fact linear.

5. It is shown that the slope of the pressure-deformation curve or 'stiffness' depends on Young's modulus and the internal pressure. It is not possible to separate these two variables by direct means.

6. A dimensional analysis shows that it is permissible to scale down from model experiments to arrive at Young's modulus and the internal pressure of the cell. For any given value of 'stiffness' there is a series of solutions, with values for the modulus decreasing from a certain figure, and values for the internal pressure increasing from nil. A limit can, however, be set to this series by other measurements.

We should like to thank Prof. Sir James Gray for many helpful discussions in the course of this work. We should also like to thank Dr D. G. Ashwell for his dimensional analysis of the cell elastimeter problem and for the suggestions which he, and Mr J. Dainty, have made about this paper. We are indebted to the Directors and staffs of the Marine Station, Millport, and the Stazione Zoologica, Naples, for their kindness and co-operation.

Some of the work described in this paper was done while the authors were at the Department of Zoology, University of Cambridge.

APPENDIX

DIMENSIONAL ANALYSIS OF THE CELL ELASTIMETER PROBLEM

By Dr D. G. ASHWELL, *University College, Cardiff*

The use of rubber models to investigate the behaviour of cells is based on the assumption that over the range of percentage elongation occurring, the material of the cell wall has a stress-strain relationship similar to that of the model. This means that the stress-strain curve for one can be derived from that for the other by changing only the scale of stress. A discussion of the likelihood of such an assumption being true is beyond the scope of this note, but it is worth pointing out, first, that there does seem to be a 'rubber-like state of matter' associated with materials capable of large elastic strains (Treloar, 1949); and secondly, that the behaviour of typical rubbers is substantially linear over the range of strains involved.

If the external dimensions of cell and model are geometrically similar, the measured deflexion of the bulge, Δ, will depend on:

(i) The diameter of the cell or model, d. This has the dimension of length, L.

(ii) The stiffness, e, of the wall of the cell or model when stretched in its own plane. This may be taken as the strain (per unit length) of a small rectangular element subjected to a tension of unity per unit length in one direction and prevented from stretching in the other direction in its plane. This has dimensions MT^{-2} (M is mass, T is time).

(iii) The flexural rigidity, f, of the wall. This is the change of curvature of a small element subjected to a bending moment of unity per unit length about one pair of opposite edges, and prevented from bending about the other. This has dimensions ML^2T^{-2}.

(iv) The pressure difference, p, between the pressure in the cell or model and that in the tube. This has dimensions $ML^{-1}T^{-2}$.

Thus Δ (whose dimension is L), may be written

$$\Delta = \Sigma A_n d^{\alpha_n} e^{\beta_n} f^{\gamma_n} p^{\delta_n}, \tag{1}$$

where A, α, β, γ and δ are dimensionless constants, and n has as many values as may be necessary.

Since (1) must be dimensionally homogeneous, for all values of n

$$L = L^{\alpha}(MT^{-2})^{\beta}(ML^2T^{-2})^{\gamma}(ML^{-1}T^{-2})^{\delta}.$$

Equating the exponents of L, M and T, and solving the resulting equations for α and δ, it is found that (1) may be written

$$\Delta = \Sigma A_n d^{(1-\beta_n-3\gamma_n)} e^{\beta_n} f^{\gamma_n} f^{\gamma_n} p^{(-\beta_n-\gamma_n)},$$

i.e.

$$\Delta = \Sigma A_n d(d^{-1}ep^{-1})^{\beta_n}(d^{-3}fp^{-1})^{\gamma_n},$$

or

$$\frac{\Delta}{d} = \phi\left(\frac{e}{pd}, \frac{f}{pd^3}\right), \tag{2}$$

where ϕ is a function to be determined experimentally.

If the symbols referring to the model are distinguished by primes, (2) states that if

$$\frac{e}{pd}=\frac{e'}{p'd'} \quad \text{and} \quad \frac{f}{pd^3}=\frac{f'}{p'd'^3}, \tag{3}$$

then

$$\frac{\Delta}{d}=\frac{\Delta'}{d'}. \tag{4}$$

If the wall of the cell may be regarded as having a constant thickness, t, a Young's modulus, E, and a Poisson's ratio, σ, it can be shown (Timoshenko, 1940) that

$$e=\frac{Et}{1-\sigma^2} \quad \text{and} \quad f=\frac{Et^3}{12(1-\sigma^2)}. \tag{5}$$

Thus (3), and hence (4), will be satisfied if

$$\frac{t}{d}=\frac{t'}{d'} \quad \text{and} \quad \frac{E}{p}=\frac{E'}{p'}, \tag{6}$$

and

$$\sigma=\sigma'. \tag{7}$$

Of these, (7) is likely to be unimportant, (4) and (6) being those required.

(4) and (6) could have been obtained more quickly and directly without introducing e and f, but should it become necessary to use models having non-homogeneous walls —if, for example, it were found that significant changes took place in the elastic properties of the internal layers only of the cell wall, (3) would be used instead of (6).

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EXPLANATION OF PLATE 10

Fig. 1. A sea-urchin egg on the end of a micropipette, showing a moderate deformation.

Fig. 2. The final version of the 'cell elastimeter'.

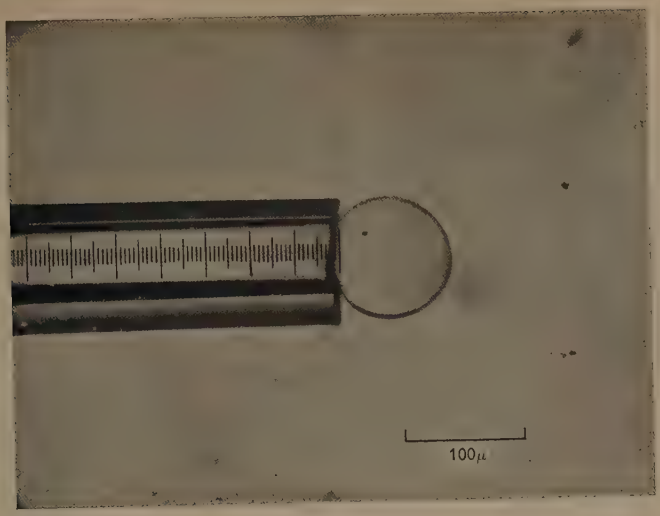

Fig. 1

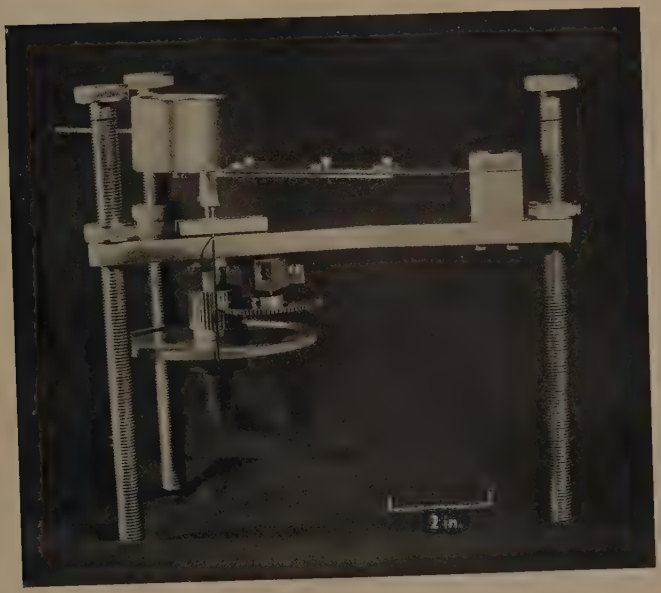

Fig. 2

MITCHISON AND SWANN—THE MECHANICAL PROPERTIES OF THE CELL SURFACE

THE MECHANICAL PROPERTIES OF THE CELL SURFACE

II. THE UNFERTILIZED SEA-URCHIN EGG

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INTRODUCTION

In the first paper of this series (Mitchison & Swann, 1954), we have discussed the problems of measuring the mechanical properties of the cell membrane and described both the construction and use of the 'cell elastimeter', and the method of analysing the results by means of model experiments. The present paper is concerned with measurements made on the unfertilized eggs of five species of sea urchin, with the object of determining Young's modulus for the membrane and the internal pressure of the cell. Future papers will describe the changes in the membrane of the fertilized egg up to the time of cleavage, and the influence of chemical agents and electric currents on the membrane of the unfertilized egg.

MEASUREMENTS ON NORMAL UNFERTILIZED EGGS

Measurements were made with the cell elastimeter on the naked unfertilized eggs of *Psammechinus miliaris* at Millport, and of the four species of sea urchin available at Naples (*P. microtuberculatus*, *Paracentrotus lividus*, *Sphaerechinus granularis* and *Arbacia lixula*). The apparatus and method are described in the first paper. The results are shown in Table 1. Except for *Psammechinus microtuberculatus*, the measurements were made on fifty eggs of each species, in batches of ten taken from five different females. The points for each batch of ten eggs were plotted on a pressure-deformation graph and the best straight line was then drawn through them. We have called the slope of this line (expressed in dynes/cm.^{2}/μ deformation) the 'uncorrected stiffness' of the cell membrane. The reasons for this are discussed in the first paper, but we should point out here that this stiffness figure is proportional to the Young's modulus of the membrane. A different procedure was adopted for the first three batches (17, 17 and 16 eggs) of *P. microtuberculatus*, in order to show the variation within a batch. A separate line was drawn for each egg, and the average and standard deviation of the slopes of these lines was calculated for each batch.

The uncorrected stiffness figures given in col. 3 of Table 1 are not strictly comparable because of the different sizes of the eggs. It has been shown in the first paper, however, that the results from elastimeter experiments can be scaled up or down, so that it is possible to produce 'corrected stiffness' values for the standard condition of a 100μ diameter egg and a 50μ diameter pipette. These are given in col. 4.

It is also possible to calculate by means of model experiments absolute values for Young's modulus from the corrected stiffness figures. These figures are given in col. 5. The methods and assumptions involved in this calculation are described in the first paper, but two points should be emphasized: the absolute values are only approximate; and they assume no internal pressure in the egg (i.e. no resting tension

Table 1. *Stiffness of the membrane of unfertilized eggs, with 50 μ diameter pipette*

(1) Species (av. egg diam.)	(2) No. of eggs	(3) Av. uncorrected stiffness (dynes/cm.^{2}/μ deformation)	(4) Corrected stiffness (dynes/cm.^{2}/μ deformation, for 100 μ diam. egg and 50 μ diam. pipette)	(5) Maximum Young's modulus (dynes/cm.^{2})
Psammechinus miliaris (105 μ)	10	7·0		
	10	8·9		
	10	7·4		
	10	10·5		
	10	10·2		
	50	8·8	8·2	$0{\cdot}91 \times 10^4$
Psammechinus microtuberculatus (110 μ)	17	8·7 ($\sigma = 1{\cdot}6$)		
	17	9·3 ($\sigma = 1{\cdot}7$)		
	16	10·6 ($\sigma = 1{\cdot}2$)		
	10	12·6		
	10	10·7		
	70	10·4	9·0	$1{\cdot}0 \times 10^4$
Paracentrotus lividus (90 μ)	10	10·1		
	10	9·1		
	10	6·6		
	10	7·9		
	10	12·8		
	50	9·3	11·1	$1{\cdot}23 \times 10^4$
Sphaerechinus granularis (100 μ)	10	8·9		
	10	10·9		
	10	13·7		
	10	14·5		
	10	13·5		
	50	12·3	12·3	$1{\cdot}37 \times 10^4$
Arbacia lixula (80 μ)	10	13·7		
	10	13·7		
	10	11·9		
	10	13·3		
	10	10·9		
	50	12·7	18·7	$2{\cdot}08 \times 10^4$

in the membrane). It was shown in the first paper that, for a given stiffness, there is a series of solutions for modulus and internal pressure, ranging from a certain value for modulus with nil internal pressure, to lower values for modulus and higher internal pressures. It is not possible to arrive at a unique solution by direct means; but various indirect methods are described in the next section which indicate that there is in fact no internal pressure.

MEASUREMENTS ON SWOLLEN AND SHRUNKEN EGGS

Having derived corrected stiffness values, the next step is to separate the opposing effects of Young's modulus and internal pressure. It seems impossible to devise a satisfactory method for measuring directly the very small pressures which might exist in the egg, and it is therefore necessary to fall back on indirect methods. One such method relies on the fact that if the internal pressure within a hollow elastic-walled ball is increased, the stiffness, measured by an apparatus such as the elastimeter, will also increase (as can be seen from the model experiments in the first paper). If, on the other hand, some of the contents are removed from within a ball in which there is an internal pressure, the stiffness will decrease, whereas if there is no internal pressure the ball will deform or wrinkle but there will be little or no change in the stiffness. This effect will distinguish between the presence or absence of internal pressure, and it can be simulated with sea-urchin eggs by placing them in hypo- and hypertonic solutions. Accordingly, a series of experiments were done with unfertilized eggs (*P. microtuberculatus*) in the following media:

		Approx. molarity
'Full hypertonic'	50 ml. sea water + 20 ml. 2M-NaCl	0·96
'Half hypertonic'	50 ml. sea water + 10 ml. 2M-NaCl	0·78
Normal sea water		0·54
'Half hypotonic'	50 ml. sea water + 10 ml. distilled water	0·45
'Full hypotonic'	50 ml. sea water + 20 ml. distilled water	0·39

The results for four batches of eggs are shown in Fig. 1, each point being the average corrected stiffness of five eggs. It can be seen that there is little or no fall in stiffness with hypertonic solutions, whereas in hypotonic solutions there is a sharp rise (except for one batch in 'half hypotonic'). This is a strong indication that there is no internal pressure, but it is difficult to set any limits of accuracy to the measurements for reasons that will be discussed on p. 466. It may be noted that in this experiment the stiffness values for the eggs in normal sea water are lower than those given in Table 1. For some unknown reason, these low values were given by most of the eggs during the period (spring 1952 at Naples) when this experiment was done. The eggs were normal in all other respects.

The high stiffness value in hypotonic solutions was maintained for long periods. The behaviour over half an hour is shown in Fig. 2, where the points are the corrected stiffness of individual eggs from one batch. The average diameter of the eggs also remained constant. There can therefore be little or no plastic flow in the membrane when under tension.

A more direct method, not needing the elastimeter, was used to set an upper limit for the initial stretch of the membrane; the existence of an internal pressure implies, of course, that there is such an initial stretch. Unfertilized eggs (*P. microtuberculatus*) were placed in hypertonic solutions of different strengths and were examined under the microscope after 20 min. in order to find the smallest degree of hypertonicity which caused a visible wrinkling of the membrane. This was found to be the 'full hypertonic' solution mentioned above (50 ml. sea water + 20 ml. 2M-NaCl). Eggs of the same batch in this solution, and in normal sea water, were also photographed,

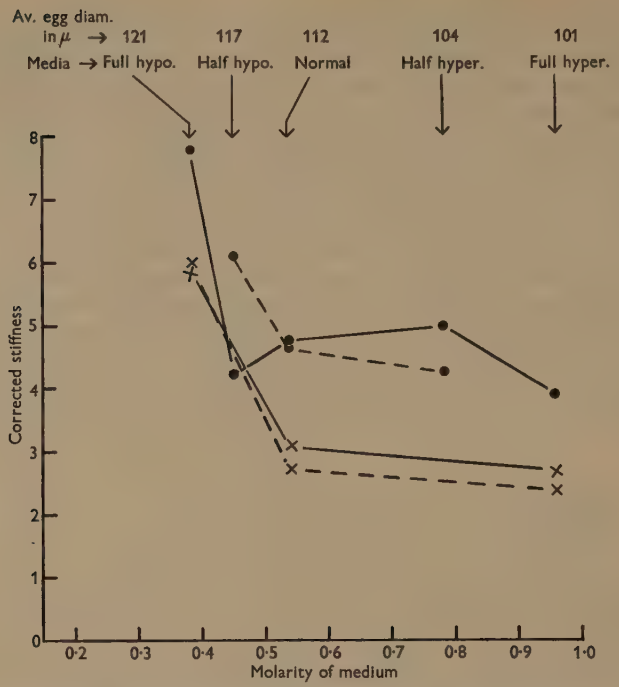

Fig. 1. Stiffness of eggs in media of different tonicity. Corrected stiffness is in dynes/cm.^{2}/μ deformation for a 100 μ diameter egg and a 50 μ diameter pipette.

and subsequently measured. Figures are given below for the average of the mean diameters (mean of major and minor axes) of fifty eggs, for the standard deviation of the mean diameters, and for the average ellipticity (major axis/minor axis).

	Average diameter (μ)	σ (μ)	Average ellipticity
Normal sea water	114	6·0	1·054
'Full hypertonic'	100	6·8	1·078

These figures show that wrinkling first occured when there had been a 12·3% shrinkage in the linear dimensions of the eggs. Since wrinkling cannot occur when there is an internal pressure, the membrane in the normal egg cannot be stretched by more than 14% (linear) from the resting state. As will be shown later, this sets an upper limit to the possible internal pressure in the normal unfertilized egg. However, it is important to emphasize that it is only an upper limit. The first signs of wrinkling are very difficult to detect under the microscope, and may occur with even smaller amounts of shrinkage than that found above. It is also more than likely that the first effect of reducing the volume of an elliptical body like an unfertilized

egg would be to increase the ellipticity rather than to produce wrinkling. Such an increase in ellipticity was in fact found in these experiments, as can be seen from the figures above.

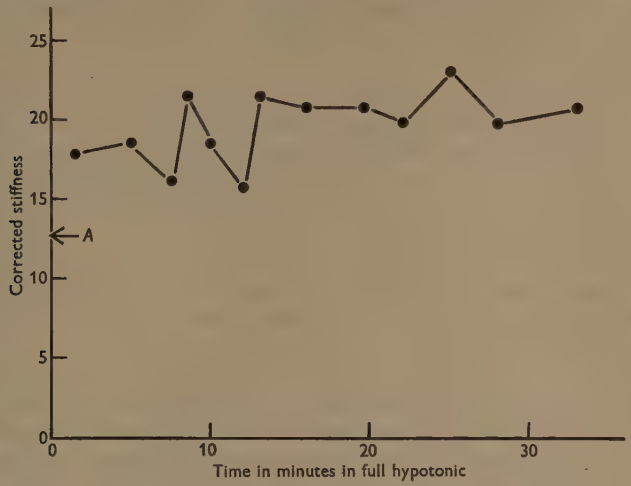

Fig. 2. Maintenance of stiffness in 'full hypotonic' medium. Corrected stiffness is in dynes/cm.^{2}/μ deformation for a 100 μ diameter egg and a 50 μ diameter pipette. *A* is the corrected stiffness value for the same batch of eggs in normal sea water.

DISCUSSION

In the first paper of this series, it was argued that the sea-urchin egg, having a membrane thickness of about 1·5 μ, resists deformation by virtue of its inherent rigidity, rather than by having an internal pressure. It is behaving therefore like a tennis ball, which maintains its shape even when punctured, rather than a rubber balloon or a fluid drop. We must now consider these arguments again, and see how far they are substantiated by the present experiments, and by earlier work.

In any experiments on the mechanical properties of the cell membrane, it is obviously important to ensure that only the membrane is being measured, and not a combination of the membrane and the cell interior. Very little is known about the elasticity of cytoplasm. Many of the demonstrations and measurements of the elasticity of 'protoplasm' are almost certainly made on the cell membrane rather than on the cytoplasm, and, where measurements have been made on the cytoplasm with centrifuge and other methods, the results are usually expressed in terms of viscosity. It seems likely, however, that normal cytoplasm is a non-Newtonian liquid with viscous-elastic properties, though its Young's modulus may be very small. The only really thorough investigation of the elasticity of cytoplasm is by Crick & Hughes (1950) who found Young's modulus of chick fibroblast cytoplasm to be about 10^2 dynes/cm.^{2}. This is only $\frac{1}{2}$–1 % of the value given earlier in this paper for the sea-urchin egg membrane. Moreover Heilbrunn (1952) concludes

that the cytoplasm of unfertilized sea-urchin eggs has a viscosity of about 3 centipoises, which is only 3 times that of water. It is unlikely that a fluid of such a low viscosity would show any elastic effects comparable with those of the membrane, so that the measurements of stiffness made with the elastimeter must reflect largely, if not entirely, the mechanical properties of the surface. Within the unfertilized egg there is, of course, the nucleus, and within the fertilized egg the nucleus and at certain stages, the asters. Both these structures almost certainly have a greater rigidity than normal cytoplasm, but the deformations produced by the elastimeter are so slight that these bodies can scarcely affect the issue. It was in fact with the object of overcoming these objections to other methods of measuring the mechanical properties of the cell surface, that the elastimeter was made in the first place.

Turning to the question of the thickness of the cell membrane, it should be said in advance that we are using the term in a wide sense to include both the permeability barrier at the surface, and the cortex. Presumably, however, most of the mechanical properties are due to the cortex or 'structural membrane' (Mitchison, 1952). After the measurements of Cole (1932), and the many microdissection experiments of Chambers, there can be little doubt that the membrane is elastic and that its degree of extensibility is so great that it must have rubber-like properties. Apart from one observation by Chambers (1938) on cleaving eggs, there is general agreement that the thickness of the membrane is about 1–2 μ. For the modulus figures in this paper we have used a model with a wall thickness equivalent to 1·6 μ in the egg. This is near enough the approximate value of 1·5 μ found by Mitchison (1955) from a number of observations on centrifuged eggs. In any case these modulus figures are not very sensitive to small changes in the membrane thickness.

The straight-line pressure-deformation curve given by the egg is further evidence of a thick membrane. It was shown in the first paper that rubber balls with an appreciable wall thickness also give a straight line, whereas solid rubber balls give a concave curve and very thin-walled balls (e.g. rubber balloons) give a convex one. Calculations show that pure surface tension would also give a convex curve.

The most difficult problem to be considered is whether or not the normal egg has a tension at the surface, and therefore an internal pressure. This question cannot be answered from elastimeter experiments alone, since the model experiments of the first paper have shown that the same stiffness can be given either by a high Young's modulus in the membrane and no internal pressure, or by a low modulus and high pressure. Nor is it possible to measure the pressure directly since the difficulties of measuring absolute pressures of the order of 10^{-5} atmosphere in a microscopic object appear at the moment to be insuperable. We must therefore turn to the indirect methods which have been described earlier in this paper.

The absence of any significant decrease in the stiffness on shrinking the eggs is a strong indication that there is no internal pressure. It does not, however, seem worth trying to be more quantitative about these experiments because of the uncertainty about the state of the membrane in hypertonic media. We cannot tell whether or not the membrane shrinks in the medium and, if it does shrink, whether

the decrease in stiffness caused by the smaller thickness compensates for the increased stiffness that would be caused by the lower hydration of the membrane material.

The experiments on the wrinkling point have the advantage that they can be used to set an upper limit on the possible internal pressure. If it is assumed that the membrane of the shrunken egg in 'full hypertonic' is unstretched and that there is no internal pressure in the egg, then the maximum possible stretch of the membrane in the normal egg is 14% (7 μ radial displacement). Using the data in text-fig. 16 of the first paper, this gives for the normal egg a maximum internal pressure of 95 dynes/cm.^{2}, with a reduction of Young's modulus from 1×10^4 dynes/cm.^{2} to $0{\cdot}54 \times 10^4$ dynes/cm.^{2}. We must emphasize that because the radial displacement is a maximum figure (p. 463), therefore this value of the internal pressure is only an upper limit. It is not an average value for the internal pressure of the unfertilized egg, nor does it imply that there is necessarily any internal pressure at all.

A further piece of evidence which indicates the absence of internal pressure is the oval shape of normal unfertilized eggs. It seems unlikely that they would maintain this shape with an internal pressure when it is remembered that they become spherical as soon as a definite internal pressure is produced by hypotonic media.

Summing up the observations on the internal pressure of the unfertilized egg, we believe that the balance of the evidence suggests the absence of such a pressure; but, if it exists, it cannot exceed about 95 dynes/cm.^{2}.

There appear in any case to be no good general grounds for believing in the existence of a resting tension in the normal cell. The idea of a tension has been popular in the past because most previous theories of the cell surface have been in terms of a surface tension whose value would be independent of the extension. Surface tension in the strict sense, however, is a phenomenon which only occurs at a liquid/liquid or liquid/gas interface, and there is little reason to suppose that the surface of a cell behaves in this way. It is clear, both from our measurements and from those of earlier workers, that most of the cell surface behaves like an elastic solid, whose tension varies from the extension and which can exist without any resting tension. The only possible place for a true liquid surface tension would be the outer boundary layer between the cell surface and the outside medium. The exact structure of this layer is unknown, but the most plausible suggestions are that it is composed either of the proteins of the vitelline membrane or the antigenic layer, or of close packed lipid molecules lying radially. Neither of these would be expected to behave as a liquid and show a true surface tension, and there is no particular reason why they should show any resting tension.

Turning to other work on the mechanical properties of the cell membrane, the most important paper to be considered is that by Cole (1932). This is an admirable piece of work, which, by a most ingenious method, gave definite proof that the behaviour of the cell surface must be governed by an elastic membrane rather than by an interfacial surface tension. Our only major criticism concerns Cole's derivation of an internal pressure of 40 dynes/cm.^{2} in the normal unfertilized sea-urchin egg. His method is briefly as follows. Eggs were compressed between a movable

flat gold leaf and a fixed parallel surface. The force (F) exerted on egg was found from the deflexion of the gold leaf, and the compression of the egg (z) was observed down a microscope. The area of contact (A) between the egg and the surfaces was calculated from drawings and photographs, and then plotted against z. F/A was found from the curves of z/F and z/A, and was equated with the internal pressure of the egg (P). Finally P was plotted against z, and, by extrapolating the curve, P was found to be 40 dynes/cm.^{2} when $z = 0$ (no compression). This method seems to us to be inherently unsatisfactory. Since the plot of $P\,(=F/A)$ against z is not a straight line, and does not follow any known theoretical formula, the extrapolation

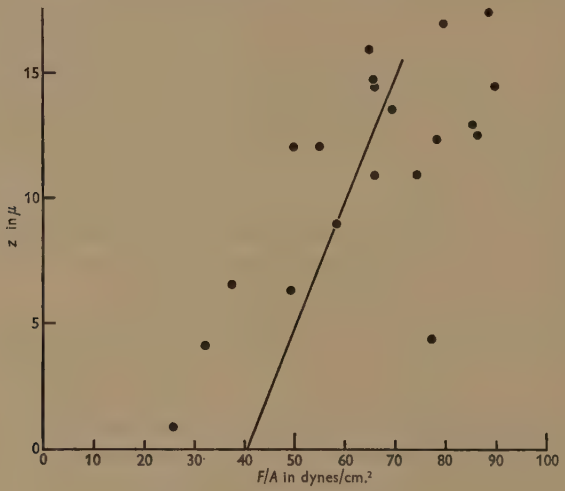

Fig. 3. Replotting of curve and points from Cole (1932).

demands an accurate knowledge of F/A for small values of z. F can probably be determined with reasonable accuracy, but A (the area of contact) is a different matter. It is varying very rapidly when the egg is only slightly compressed (up to 300 μ^2 per 1 μ compression) and is in any case a very difficult figure to measure or calculate. Small errors in A will therefore make large differences in the extrapolated value for the resting internal pressure. This also implies that smoothing of the curves should be used with considerable discretion, which raises another difficulty with Cole's paper. Cole draws a reasonable smooth line through the points in his z/A graph, which when combined with the z/F graph, gives the curve for F/A $(=P)$ against z which is reproduced as the *curve* in Fig. 3. If, however, the first twenty points on the z/A graph (i.e. those for small compressions) are used instead of the smooth line, they give, after conversion, the *points* reproduced in Fig. 3. These points show a large scatter and it is doubtful whether any extrapolation is justified, but, if this were to be done, the curve would cut the abscissa much nearer the origin

than Cole's curve. If the three lowest points (where the largest error is likely) were ignored, it might well be taken to pass through the origin, and to indicate no internal pressure.

Apart from these particular criticisms of Cole's figures, there is a serious objection to the use of this method of finding resting pressures when dealing with comparatively thick-walled and rigid membranes. In a structure like a tennis ball the main resistance to small indentations comes from the bending of the wall near the indentation. Cole's calculations, however, assume a thin elastic membrane where the resistance comes from the increased internal pressure due to the general stretching of the walls. The greatest error will occur in the calculations for small deformations, which are the critical ones for the determination of the resting pressure. In consequence, as Cole himself pointed out in a later paper, the internal pressure determined in this way will not be correct when the measurements are made on a rigid membrane (i.e. an elastic one of appreciable thickness).

For these reasons, we believe that Cole's figure of 40 dynes/cm.^{2} for the internal pressure (or the figure of 0·08 dyne/cm. for the surface tension which follows from it) is of doubtful value, and we have not felt justified in using a similar method to calculate internal pressures from the elastimeter figures. It is possible that one could get accurate information from Cole's method if it was used together with model experiments similar to those with rubber balls that we have described earlier. However, we think that the elastimeter is a more satisfactory method since it is easier, quicker, and deforms the egg much less. It is worth pointing out that the largest compression of the egg by Cole's method increases the surface area by about 50%, whereas the maximum increase of surface area with the elastimeter is only about 5%.

Most of the other experiments on the surface forces in cells have been reviewed by Harvey & Danielli (1938). All of them suffer from the defect that they measure tension at a single point when the cells are deformed or stretched. This is adequate when dealing with a true surface tension which does not vary with extension, but in the case of an elastic body it is not very informative since it only gives one point on the tension/extension curve, and neither the elastic modulus nor the resting tension, if any, can be found from it. One of the most widely used methods has been to measure the force necessary to split cells in two with a centrifuge, and it is satisfactory to find that when applied to sea-urchin eggs the results are comparable with those given in this paper. Harvey (1931) found in unfertilized *Arbacia pustulosa* eggs that this method gave a surface tension of 0·2 dynes/cm. when the surface had been increased by 25%. Using the data in text-fig. 16 of the first paper, and assuming the modulus of 2×10^4 dynes/cm.^{2} found for *A. lixula*, an area increase of 25% would give an internal pressure of 180 dynes/cm.^{2}. From the normal surface tension relation $P = 2T/R$, this pressure would be produced by a surface tension of 0·36 dynes/cm. If the modulus in the American species of *Arbacia* was 1×10^4 dynes/cm.^{2} (a value nearer to that we have found for the other sea urchins) the surface tension at this extension would be 0·18 dyne/cm. Considering the difference in the methods and assumptions, the agreement with Harvey is

reasonable. It must be remembered, however, that neither this, nor any of the comparable experiments, provide evidence that there is any tension and hence internal pressure in the *undeformed* cell.

In the light of the cell elastimeter experiments, and the discussion above, it is evident the unfertilized sea-urchin egg is bounded by a solid elastic membrane, and filled with fluid or nearly fluid cytoplasm. Under normal conditions there is probably no internal pressure (and therefore no tension in the membrane) but if there is a pressure, it does not exceed 95 dynes/cm.^{2}. The thickness of the membrane is about 1·5 μ (1–2 % of the diameter), and it has a Young's modulus of the order of $1–2 \times 10^4$ dynes/cm.^{2}. There is no great difference between the different species of sea urchin, but *Arbacia* has a rather higher modulus ($2{\cdot}08 \times 10^4$) than the other species ($0{\cdot}91–1{\cdot}37 \times 10^4$). For comparison, some other values of Young's modulus are (in dynes/cm.^{2}): steel, 2×10^{12}; rubber, about 10^7; muscle (static), $0{\cdot}5 \times 10^6$ (Buchthal & Kaiser, 1951); Myxomycete threads, 9×10^4 (Norris, 1940); chick fibroblast cytoplasm, 10^2 (Crick & Hughes, 1950). A modulus of 10^4 shows that the egg membrane is not a very rigid structure. It would have a consistency similar to a weak table jelly, but, in an object as small as a sea-urchin egg and only slightly denser than its surrounding medium, this degree of rigidity is sufficient to ensure that the cell maintains its shape even when there is no internal pressure (as in hypertonic media). In everyday terms, the egg resembles a tennis ball or a child's rubber ball, rather than an inflated balloon or an oil drop in water.

NOTE ON THE EFFECT OF TEMPERATURE

The action of various chemical and physical agents on the mechanical properties of the cell membrane will be described in subsequent papers, but it seems appropriate at this point to describe some experiments on the effect of temperature on stiffness. Measurements were made on unfertilized eggs (*Psammechinus microtuberculatus*) at room temperature, and at a temperature of 3° C. in a cold room. Each of the figures below is the average corrected stiffness for five eggs.

21·5° C.	3° C.
9·8	16·5
9·1	22·0
8·1	20·9
Av. 9·3	Av. 19·8

These figures show an average increase in stiffness by a factor of 2·1 for a drop in temperature of 18·5° C.

One characteristic phenomenon with the eggs at low temperature is the appearance of the 'yield point' mentioned in the first paper. When the bulge in the pipette is nearly hemispherical, it often appears to give way suddenly and move up the pipette. If the pressure is not released at once the whole egg may be sucked up the pipette; even so it does not cytolyse and recovers its normal shape if released.

Although it is beyond the scope of this paper, it should be mentioned that we have found a similar increase of stiffness on lowering the temperature with fertilized eggs.

These effects are unexpected and interesting. A rubbery substance normally shows a fall in Young's modulus with a fall in temperature (until crystallization takes place), whereas the egg membranes show a relatively large rise with falling temperature. Until

more is known about this temperature effect and the physical properties of the membrane material we can only speculate, but it is tempting to suggest that the stiffness is controlled by a living or enzyme-determined process which is slowed down at low temperatures. If so, this process must be working to keep the stiffness low, and the 'dead' membrane should therefore be stiffer than the 'living' one. There is some evidence that this is the case.

Marsland (1950) has measured the effect of temperature on the 'structural strength' of the cortical gel of unfertilized *Arbacia* eggs. He takes as a measure of this 'structural strength' the time required with a given centrifugal force to produce a standard stratification of the pigment granules from the cortex. At first sight his results would seem to be at variance with our own, since he finds a fall in the 'structural strength' with falling temperature, but it is doubtful how far these two sets of results are comparable. In the first place, Marsland's figure is really a measure of the viscosity of a fluid, and not of the elasticity of a solid. When applied to a solid, it is uncertain exactly what physical property would be measured, but it is possible that the yield point would be involved, and we have shown that there is some evidence that this changes with temperature in the opposite way from stiffness. There is a second, and more serious objection to Marsland's experiments which has been pointed out by Wilson (1951). Marsland assumes that the pigment granules in the unfertilized egg are located in the cortex. There are, in fact, a number of these granules in the cortex but there are also a large number in the cytoplasm, and it is only after fertilization that the majority of the granules move into the cortex (Harvey, 1910). This is shown by a count of the granules in the surface made with a high-power water-immersion objective in green light (to render the red granules conspicuous). An unfertilized *Arbacia punctulata* egg has about twenty granules per $100\,\mu^2$ of surface, whereas the fertilized egg has about fifty. There is no evidence that the number of granules changes at fertilization, so the majority of the granules in the unfertilized egg must be in the cytoplasm.

This implies that one of the components of Marsland's figures may be the cytoplasmic viscosity. It seems unlikely, however, that it can be simply this viscosity because Costello (1934), using yolk granule stratification, found that the cytoplasmic viscosity *increased* about 3 times on a drop of temperature from 20 to 30° C. Costello also pointed out that the pigment granules behaved in a different way from the yolk granules. Whereas the latter gave an apparent viscosity rise, the former would have given a constant viscosity or a slight fall. This has been taken by Marsland to be a confirmation of his views on the cortical strength, but it may well be that one or other of the types of granule has different physical properties (e.g. size or density) at different temperatures. It is worth remembering that Harris (1939) showed that the pigment granules behaved more like vacuoles than solid granules.

It may be noted that Norris (1940) found a temperature effect on the Young's modulus of Myxomycete threads which is very similar to our own results. The modulus increased by a factor of about two for a drop in temperature of 14° C. (from 24 to 10° C.).

SUMMARY

1. Measurements were made with the cell elastimeter on the stiffness of the cell membrane in the unfertilized eggs of five species of sea urchin. Young's modulus varies in the different species between the values of $0{\cdot}91 \times 10^4$ and $2{\cdot}08 \times 10^4$ dynes/cm.^{2}.

2. Experiments on the change of stiffness in hypo- and hypertonic media indicated that there is probably no internal pressure and no membrane tension in the normal

egg. If, however, there is an internal pressure, measurements of the minimum shrinkage of the membrane necessary to produce wrinkling showed that this pressure cannot exceed 95 dynes/cm.^{2}.

3. A drop in temperature of 18·5° C. produced an increase of stiffness by a factor of 2·1.

4. These experiments, together with other evidence, suggest that for mechanical purposes the unfertilized sea-urchin egg can be compared to a hollow sphere filled with fluid and surrounded by a solid elastic wall (the membrane or cortex) about 1·5 μ thick and with an elastic modulus about $1\text{–}2 \times 10^4$ dynes/cm.^{2}. This degree of rigidity is sufficient to ensure the maintenance of shape of the egg without the presence of an internal pressure or a tension in the membrane. In everyday terms, the egg therefore resembles a tennis ball or a child's rubber ball, rather than an inflated balloon or an oil drop in water.

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